RE-EXAMINING TEMPORAL AND SEASONAL MICROBIAL ACID MINE DRAINAGE COMMUNITY VARIATION

By

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science (MSc) in Chemical Sciences

The School of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

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THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
School of Graduate Studies/École des études supérieures

Title of Thesis Titre de la thèse	RE-EXAMINING TEMPORAL AND SEASONAL MICROBIAL ACID MINE DRAINAGE COMMUNITY VARIATION
Name of Candidate Nom du candidat	Auld, Ryan Richard
Degree Diplôme	Master of Science
Department/Program Département/Programme	Chemical Sciences
Date of Defence Date de la soutenance	November 14, 2013

APPROVED/APPROUVÉ

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Abstract

Acid Mine Drainage (AMD) is characterized by high metal concentrations and an extremely low pH, primarily generated by the microbial oxidation of iron sulfides from mine tailings. Research on the microbial AMD community has largely focused on Bacteria, while little information is known about the Archaeal and Eukaryote members or the seasonal patterns within the communities. Here I examined the Bacterial, Archaeal, and eukaryotic AMD seasonal microbial community, using direct sequencing techniques on AMD samples from the Copper Cliff Tailings AMD site in Sudbury, Ontario, Canada. I found large variation in the community profile and species composition between sampling times of both the Bacterial and Eukaryote communities, suggesting a dynamic community, both between and within seasons. Bacterial diversity was highest during the winter, with *Acidithiobacillus* dominating, while during the summer, *Acidiphilium* was the dominant genus. The winter Eukaryote community was dominated by classes of algae and fungi, while the majority of summer sequencing could not be classified to the class level. Few reads were obtained for the Archaeal domain, with low and similar biodiversity between seasons. Overall, the AMD community variation and abundance were found to largely correlate with drainage water and seasonal temperature.

Keywords

Acid Mine Drainage, 454 Pyrosequencing, Prokaryote, Eukaryote, Community.

Co-Authorship Statement

Chapter 2 was co-authored by Nadia C. S. Mykytczuk, Leo G. Leduc, and Thomas J. S. Merritt.

Acknowledgments

I would like to express my gratitude, foremost to Dr. Thomas Merritt, for giving me the opportunity to undertake Graduate School, as well as for his guidance, expertise, and friendship. I also gratefully thank Dr. Nadia Mykytczuk, and Dr. Leo Leduc for taking me on and allowing me to work with them over the last several years.

I would like to thank my parents, sister, and family for their continued support and guidance throughout my studies. I would not be where I am today without the help from my family.

I thank David Bing, Jose Knee, and Teresa Rzezniczak, an amazing group of friends I made during graduate school, for their assistance and for making life more interesting. I thank Jordan Mathieu for his constructive comments and friendship along the way. I would also like to especially thank my closest friend, Janique Vandal, for her support and assistance with Microbiological techniques during my Masters.

I would like to thank my committee members, Dr. Eric Gauthier, and Dr. Mehrdad Hajibabaei for taking the time to provide great feedback and support along the way.

I also thank Glen Watson and Vale Inc. for allowing us on their site to perform sampling, as well as for their support and financial contribution to this project.

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List of Abbreviations

Abbreviation	Definition
At. ferrooxidans	*Acidithiobacillus ferrooxidans*
AMD	Acid mine drainage
A. acidophilum	*Acidiphilium acidophilum*
nBLAST	Nucleotide basic local alignment search tool
DGGE	Denaturing gradient gel electrophoresis
FeRB	Iron reducing Bacteria
FISH	Fluorescent *in situ* hybridization
LDS	Low density sludge
L. ferrooxidans	*Leptospirillum ferrooxidans*
OTU	Operational taxonomic unit
PCoA	Principle coordinate analysis
RDP	Ribosomal database project
Read	Direct pyrosequencing sequence
RMT	Rapid mix tank
rRNA	Ribosomal ribonucleic acid
SRB	Sulfate reducing Bacteria
ug/L	Micro-grams per liter
MEND	Mine Environment Neutral Drainage (MEND) program
mg/L	Milli-grams per liter
uS/cm	Micro-siemens per centimeter
Qiime	Quantitative insights into microbial ecology

Chapter 1

1 Introduction

1.1 Acid Mine Drainage

With a global increase in mining related activity (Palmer et al. 2010), increased attention is being paid to the effects of mining on the environment. Acid mine drainage (AMD), produced through chemical and microbial oxidation of sulfide minerals from mining wastes, is considered the largest environmental problem facing the mining industry (Egiebor and Oni 2007). The mine wastes (tailings) produced from industrial smelting are often collected on large open sites. It is estimated that 7 billion tons of mine tailings covers about 41,000 hectares of land in Canada, from which the estimated cost of remediation is between 2 to 5 billion dollars depending upon the method of treatment (MEND 1994). These tailings are not chemically stable and minerals are leached out of the tailings by percolating water runoff. This drainage water contains high concentrations of iron, sulfides, and associated metals, and is inhabited by an array of chemolithotrophic organisms, which obtain their energy by oxidizing sulphide and related minerals.

Several chemical reactions take place in the process of drainage acidification, and the nature of these reactions differ depending on water geochemistry and the minerals being utilized by the microbes. Pyrite is the most abundant sulphide mineral on the planet (Johnson and Hallberg 2005), and is often used to illustrate the oxidation reactions that

take place in the production of AMD. Different AMD microorganisms utilize different minerals and ions for energy. Some organisms use reduced inorganic compounds (chemolithotrophy) while other can use organic carbon sources (heterotrophs). Initially the sulphide minerals (pyrite shown below) are oxidized to ferrous iron, sulfate and hydrogen (equation 1). This initial oxidation is an abiotic reaction occurring when sufficient oxygen and moisture are present. In addition, Bacterial oxidation of pyrite to ferrous iron species is also proposed to occur by sulphur oxidizing neutrophiles, such as *Thiomonas* and *Thiobacillus* species, followed by further acidification by sulphur oxidizing acidophiles such as *Acidiphilium* species (Leduc et al. 2002).

Equation 1

$$FeS_2 + 7/2O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + H^+$$

Oxidation of ferrous iron to ferric iron (equation 2) is controlled completely by Bacterial activity and has been proposed to be the rate limiting step in the production of AMD (Singer and Stumm 1970). This step is mainly controlled by iron oxidizing acidophiles, predominantly *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, both of which have received considerable attention due to their role in acidification (Edwards et al. 1999; Leduc et al. 2002).

Equation 2

$$Fe^{2+} + 1/4O_2 + H^+ \rightarrow Fe^{3+} + 1/2H_2O$$

At low pH, the ferric iron reacts with hydroxide in the drainage to produce iron hydroxides, such as $Fe(OH)_3$, which will precipitate, further reducing the drainage pH

(Akcil and Koldas 2006). Ferric iron that does not precipitate oxidizes additional pyrite to form more ferrous iron (equation 3), which is again oxidized by iron oxidizing acidophiles in equation 2.

Equation 3

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$$

During the additional oxidation of pyrite by ferric iron, elemental sulphur is produced (not shown in equation 3) which is utilized by sulphur oxidizing acidophiles such as *Thiobacillus* species (equation 4).

Equation 4

$$2S^0 + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$$

The production of AMD is not solely the result of the pyrite dissolution and oxidation. Although pyrite is the most widely studied, a wide range of sulphide minerals and reactions likely contribute to the production of AMD. Other sulfide minerals, such as pyrrhotite, are utilized with similar metabolic pathways (Nicholson et al. 1994), but less is known about their oxidative processes. Pyrrhotite is the second most common sulphide in nature (Belzile et al. 2004) and is more susceptible to chemical and Bacterial oxidation than pyrite (Pinka 1991). The chemical and physical characteristics of AMD sites are dependent upon the minerals being mined and mineral deposits of the area (Lottermoser 2003). This variability in the geochemistry and physical properties of AMD sites has been proposed to correlate with the microbial variation seen at AMD sites (Kuang et al. 2013; Baker and Banfield 2003). Current research is underway to explain the variability

of geochemical and physical properties and how these properties affect the microbial communities. The site pH has been proposed to be a major influence on the AMD microbial community (Kuang et al. 2013), while factors including rainfall and conductivity have also been implicated (Edwards et al. 1999).

The oxidation of ferrous to ferric iron has been identified as the rate determining step in drainage acidification (Singer and Stumm 1970) and *At. ferrooxidans* and *L. ferrooxidans* species are implicated in the control of this crucial step in AMD production (Schrenk et al. 1998). *At. ferrooxidans* and *L. ferrooxidans*, appear to have similar roles in iron oxidation, with one species dominating the other according to the environmental conditions (Sand et al. 1992; Schrenk et al. 1998; Edwards et al. 1999). Specifically, *At. ferrooxidans* has been found to dominate in higher pH environments (1.5 – 2.4) (Edwards et al. 1999; Schrenk et al. 1998), while *L. ferrooxidans* is often isolated from AMD biofilms and even lower pH sites (Schrenk et al. 1998; Bond et al. 2000). More recently, several different microorganisms such as Archaeal *Ferroplasma* spp., and several Bacterial species including *Leptospirillum* group III, *Gallionella ferruginea* and *Ferrovum* spp., have been found as dominant organisms in a range of AMD sites, and are suspected to also play a role in oxidation of ferrous iron (Bond et al. 2000; Hallberg et al. 2006; Tan et al. 2009; Huang et al. 2011). The Archaeal *Ferroplasma* genus which has been isolated from extremely low pH sites (0 – 1.7) is receiving considerable attention due to its high abundance in some AMD systems and potential iron oxidative role (Bond et al. 2000; Dopson et al. 2004). Initially believed to be a simple system, further

characterization of AMD sites and of these more recently described AMD microorganisms is expanding our understanding of AMD.

Advances in culture independent environmental characterization techniques are identifying large variation in AMD microbial community composition between sites and seasons. These results are suggesting that acidophilic organisms, which were originally thought to play minor roles in AMD environments and in the production of AMD, may actually play more significant roles. Recently, direct pyrosequencing of the AMD Bacterial domain at Vale's Copper Cliff central tailings facility (ON, Canada) revealed that neutrophilic *Legionella* species were the second most abundant genus (Auld et al. 2013). This result was surprising in that *Legionella* had been documented at only one other AMD site in China (Hao et al 2010). How this generally neutrophilic group has adapted to the acidic environment is still unexplained. In addition to *Legionella*, our previous work found the Copper Cliff AMD site to be dominated by *Acidithiobacillus* spp. as well as several more rare organisms, previously unknown or poorly documented at AMD sites such as *Halomonas*, *Alicyclobacillus*, and *Granulicella* spp. (Auld et al. 2013). All in all, these unanswered questions highlight the need for a more detailed analysis of the AMD microbial community.

1.2 Temporal and Seasonal AMD Microbial Community Trends

The AMD microbial community appears to vary with seasons, but previous work is limited. Previous research using culture-based approaches to characterize the AMD community suggested seasonal variation does exist among the Bacterial community (Leduc et al. 2002). Similarly, culture-independent techniques also revealed seasonality in the AMD microbial community although the majority of this work relies on examining a few key taxa to describe community trends (Edwards et al. 1999). Research into the entire seasonal AMD microbial community (Bacteria, Archaea, and Eukarya) and its variation is lacking, and to my knowledge, no previous work has been done to assess the seasonality in the microbial AMD community using direct sequencing.

Previous characterization of seasonal variation in the microbial AMD community has utilized culture-dependant or molecular, fingerprinting techniques. These techniques are known to be biased since the majority of microbes cannot be cultured (Amann et al. 1995), and return a less complete community profile than more recent high-throughput/direct sequencing, respectively. Molecular techniques suggest that the complete seasonal microbial AMD community is largely composed of Bacteria, although Archaea may represent a sizable, even dominant, fraction of the population, in some specific locations or sites with high conductivity, high metal concentrations, or extremely low pH (Tan et al. 2009; Edwards et al. 1999).

The two major Bacterial iron oxidizers often implicated in AMD production, *Acidithiobacillus* and *Leptospirillum*, were previously found to vary seasonally at Iron

Mountain (California, USA), with varying abundances thought to be related to AMD geochemistry (Edwards et al. 1999). The dominance of the Bacterial community, but the relatively low abundance of *Acidithiobacillus* and *Leptospirillum* species, suggests that other Bacterial species may fill the iron oxidizing role. The past's exclusive focus on these two groups may have caused researchers to miss Bacteria diversity and potentially novel organisms playing substantial roles in AMD.

These seasonal community studies, along with site specific studies, have found the AMD community variation is correlated with, and may be driven by, changes in seasonal geochemistry and physical properties (Strenten-Joyce et al. 2013; Tan et al. 2009; Edwards et al. 1999). The patterns of community change are likely related to both the available resources in, and the physical properties of, the AMD throughout the year. One goal of AMD research is to understand seasonal and site specific microbial community variation as well as the role of each community member to better describe and understand the acidification process carried out by microbial oxidation..

1.3Conventional Acid Mine Drainage Treatment Methods

Many different treatment options and methods currently exist for AMD cleanup and remediation, the most common being the alkalinization (neutralization) to remove metals from the drainage (Johnson and Hallberg 2005). Lime neutralization is by far the most widely used treatment method due to its ease of use, and the low cost of lime in

comparison to other chemical treatments (Johnson and Hallberg 2005). Although, a variety of basic compounds are also used to neutralize AMD aside from lime, including calcium carbonate, sodium carbonate, sodium hydroxide, and magnesium oxide.

Precipitation of metals from the drainage occurs when the pH is raised to the point at which the metals are insoluble, and increasing the pH of the drainage to about 9.5 will precipitate the majority of the metals (Kalin et al. 2006; MEND 2005). The difference in specific pH precipitation of certain metals is an important factor in many of the AMD treatments and processes. Ferrous iron (Fe^{2+}), for example, precipitates at pH 8 to 9 and ferric iron (Fe^{3+}) at pH of 3 (Kalin et al. 2006). Because of this difference in precipitation pH, both the method, and degree of alkylation, will determine which metals are precipitated and the efficiency of metal precipitation.

Processing of AMD at the Copper Cliff location (the location of my thesis research) is performed using a mechanically agitated reactor (MEND 2005) in which the drainage is mechanically mixed with lime to increase the pH. The mechanically treated discharge is then sent to a settling pond where AMD wastes are accumulated and the sludge can settle for solid separation (3-10% solids produced) (MEND 2005). Some treatment systems do not utilize a mechanical agitated reactor to mix the AMD and lime; rather the wastes enter a stream and a settling pond where lime treatment is directly used. Direct lime treatment in settling ponds is, however, much less efficient (50% less) than mechanically mixed systems (Aube 2005) and typically produces low density sludge (LDS), comprised

of 1-5% solids (MEND 2005). More complex AMD processing techniques exist that produce higher percentage solid separation which utilizes a rapid mix tank (RMT), (not used at the Copper Cliff site). Many different RMT configurations exist, but the overall process is similar to LDS processing, and attempts to better control the pH of the mixture (sludge/drainage) and produce a higher percentage of solid removal (15 – 30%, MEND 2005). The rapid mix tank includes a separate tank for the addition of sludge and acidic drainage with lime where the pH is monitored. Often, an additional tank is used, where air is pumped into the system to allow for ferrous iron oxidation to ferric iron which precipitates at a lower pH as explained above. The RMT often utilizes a flocculant tank in which a flocculant (chemicals used to aggregate suspended minerals) is used to bind the suspended solid metals. The mixture is then sent to another tank, called the clarifier, where the solid sludge is removed and a portion of the lime mixture is recycled (Kalin et al. 2006; MEND 2005). The use of neutralizing agents such as lime is not a long term solution to the production and remediation of AMD due to the on-going treatment needed, as AMD in continually being produced at mining sites. The classical neutralization method while quick and simple to perform, has several disadvantages including cost of chemicals, continued treatment, and need for additional sludge treatment methods, making research into new treatment methods such as bioremediation important.

1.4 Acid Mine Drainage Bioremediation

Due to the continued production of AMD, novel remediation methods are being investigated which could potentially have a sustainable long term impact, unlike neutralization processes. Bioremediation is one such approach, and employs microorganisms to increase the alkalinity and immobilise metals commonly found in AMD sites (Johnson and Hallberg 2005). The possibility of bioremediation techniques being used in AMD systems first relies on a better understanding of the complete AMD community.

Bioremediation is a new field, but has already been used successfully in cases including, oils spills, soil remediation and in AMD (Boopathy 2000; Guimaraes et al. 2010; Kalin and Caetano Chaves 2003) Bioremediation has been utilized on large scale AMD cleanup efforts, one example being the "acid reduction using microbiology" (ARUM) system. The ARUM system utilizes two oxidation cells which precipitate iron, generate sulphides, as well as increase pH, and has been found effective at several sites (Kalin and Caetano Chaves 2003). The use of microorganisms in AMD cleanup is potentially less costly, and a more efficient alternative to reducing acidity and immobilization of metals (Johnson and Hallberg 2005).

AMD sites contain high concentrations of iron and sulphate, where anaerobic sulphate reducing Bacteria (SRB) and iron reducing Bacteria (FeRB) can, therefore potentially remediate such sites as long as there is sufficient supply of organic matter for the reducing Bacteria. Specifically, sulphate reducing Bacteria have received considerable

attention in AMD remediation (e.g. Kolmert and Johnson 2001; Garcia et al. 2001) due to their ability to reduce sulphate (SO_4^{2-}) to sulphide (S^{2-}). Sulphides (S^{2-}) can then react with additional metals such as iron and copper, precipitating these metals out of solution (Kolmert and Johnson 2001). Bacteria SRBs increase the alkalinity during the oxidation of organic material or molecular hydrogen. The lack of organic matter in AMD sites may pose a problem for *in situ* AMD bioremediation due to lack of carbon sources. For such large scale bioremediation projects to be possible, the presence of acidophilic SRB is necessary and while acidophilic SRB have in the past been difficult to isolate from AMD systems it is thought to be due to lack of appropriate substrates (Johnson 2006). In addition to AMD SRB, an understanding of the *in situ* microbial community interactions and environmental properties will also be pivotal.

1.5 DNA Barcoding

DNA barcoding can be used to characterize a microbial community, and is often a first step in environmental studies. DNA barcoding is the taxonomic classification of an organism using a short DNA marker sequence. The sequences have specific characteristics. They must be short enough for DNA sequencing techniques, variable enough for classification, while still being conserved enough to allow for the production of universal primers for amplification or direct sequencing (Patel 2001).

The 16S ribosomal small subunit (rRNA) gene found in prokaryotes is one of the most widely used genetic markers in DNA barcoding (Janda and Abbott 2007). While the 18S rRNA gene, the eukaryotic homologue of the 16S rRNA gene, is also widely used (Xie et al. 2011). The 16S and 18S rRNA genes have the unique traits stated above to make them excellent marker sequences (Patel 2001; Xie et al. 2011). The 16S rRNA gene is roughly 1,500 bp long, while the 18S rRNA gene is somewhat more variable at between 1,500 – 4,500 bp long (Patel 2001; Xie et al. 2011). Additionally, the rRNA genes (i.e. both 16s and 18s) contain variable (V) regions and conserved regions (Woese 1987). "Universal" primers can be designed from the conserved regions for use in PCR and direct sequencing. The variable regions, which have larger amounts of sequence change, can be used for the identification of organisms. These two sets of regions make rRNA's important tools in microbial evolution and ecology research (Tringe and Hugenholtz 2008). The majority of sequence variation in the 18S rRNA gene is in three variable regions: the V2, V4, and V7 regions (Neefs et al. 1991). The majority of variation in the 16S rRNA gene is in nine variable regions V1 – V9. No single 16S rRNA variable region is capable of distinguishing between all Bacterial species (Chakravorty et al. 2007) and thus the variable region examined is often selected based on the community being examined.

Interestingly, marker genes (e.g. 16S and 18S rRNA genes) have been found reliable in classification of Bacteria to the genus level using 400bp sequences (Wang et al. 2007) and recent advances in next generation pyrosequencing methods (methods used in this study) can now produce reads upwards of 500bp, allowing for greater resolution of

environmental communities. This increased sequence length now allows us to classify environmental direct sequencing to the genus and even species level for high resolution community information. Previously the 100-200bp reads produced by pyrosequencing were often only utilized for phylum classification and identification of overall community changes.

1.6 DNA Barcoding Bias

The use of rRNA barcoding to examine community structure is one of the most used tools to assess microbial diversity. In being such a prevalent tool several biases exist that need to be addressed in order to understand the usefulness and limitations of this technique. Two major areas of bias are: effectiveness of DNA extraction/technique and selection of primer sets (primer bias). The use of poor DNA extraction techniques and only one primer set was found in some cases to miss up to 50% of the community diversity (Hong et al. 2009).

The quantity, quality, and techniques used to extract DNA and rRNA for direct sequencing will all affect the number of OTU's and the community profile obtained (Martin-Laurent et al. 2001). The more effective DNA extraction methods will provide more complete community analysis. Additionally, the use and choice of universal primers for assessing community members has been found as one of the most important aspect of identifying community members and overall community profiles (Klindworth et

al. 2013). Primer sets not optimized to specific environments or lineages can cause certain species to be missed or underrepresented (Baker et al. 2003). These biases associated with rRNA barcoding do not render this technique useless, but their affects need to be understood. The use of one primer set for assessing microbial diversity may in some environments miss some diversity and at times may be useful to use additional primer sets. Here we did not attempt to assess the complete microbial diversity at the Copper Cliff AMD site but direct sequencing was done as a comparative analysis with the same primer sets and extraction techniques in order to determine broad community profile changes. Diversity was also assessed but with an understanding that the use of one primer set undoubtedly misses members of the community.

1.7 Community Characterization Techniques

Our understanding of microbial communities has greatly increased with the use of culture independent community characterization techniques (Mohapatra et al. 2011). While cultivation of environmental microbes still provides researchers with biologically relevant information, the inability to culture the majority of microorganisms (Amann et al. 1995) limits the utility of culture-based techniques for describing microbial communities. Classically, in order to study a microorganism, it needed to be isolated from a complex mixture of microbes using selective media. It has been estimated that environmental culturing methods miss the majority of native organisms, up to 99% using standard culture techniques (Amann et al. 1995). In contrast, it has been found that culturing techniques work extremely well on AMD systems, capturing the majority of the predominant members of the community (Auld et al. 2013), although culturing does not

provide information on species abundance and has limited resolution of the diversity of rare species. To address these limitations in culture dependant methods, many culture independent methods arose to examine the diversity of environmental samples. Here I will only review several commonly used community characterization techniques.

Prior to the beginning of the 21st century, the majority of culture independent methods used PCR based techniques; amplifying rRNA's to examine microbial communities without sequencing. A common method, denaturing gradient gel electrophoresis (DGGE), amplifies marker genes and separated the amplified products by gradient gel electrophoresis, the amplified DNA is then stained to provide a banding pattern that is characteristic of the community (Muyzer and Smalla 1998). The process of DGGE involves the extraction of DNA from environmental samples and amplification of a marker gene, often the 16S rRNA. The amplified products are run on a gradient denaturation gel and stained to produce a unique banding pattern characteristic of the community. The gradient gel contains chemical denaturant that increases in concentration along the gel that causes unique dsDNA migration. The distance of migration of the amplified DNA sequences is controlled by the base composition.

AT regions, become more denatured than GC because of the hydrogen bonding difference between A-T (2 H-bonds) and G-C (3 H-bonds) base pairs. The increased hydrogen bonding of GC rich sequences results in more compact fragments and further gel migration (higher chemical denaturant concentration) as compared to AT rich

sequences with less hydrogen bonding. This difference in migration distances based on sequence content allows for unique species-, or environment- (compiled species pattern), specific banding patterns that can be compared between communities. Dominant community members can be identified by excision and sequencing of specific bands. This process can allow for identification of selected taxa, but not as exhaustive a list as from cloning or direct sequencing (Forney 2004). DGGE has several limitations that often make complete characterization of complex communities difficult. The analysis of DGGE gels may be very complex due to high biodiversity environments which increase the number of bands. Additionally DGGE experiments are sensitive to small spatial sampling changes due to high spatial community variation (Muyzer and Smalla 1998). Another difficulty in DGGE is the often uncorrelated relationship between band intensity and species abundance due to multiple rRNA copies found in some species, and variability in the rRNA sequences between organisms making dominant members difficult to identify (Nubel et al. 1997).

Non-PCR based methods, such as fluorescence *in situ* hybridization (FISH), have also been developed to study environmental biodiversity (Bottari et al. 2006). FISH has been extensively used in the community characterization, and specifically, temporal and seasonal AMD community variation (Schrenk et al. 1998; Edwards et al. 1999). The FISH technique utilizes fluorescently labelled oligonucleotide sequences to bind complimentary DNA or RNA sequences of a particular group of organisms (Mohapatra et al. 2011). The 16S rRNA sequence is often used due to the high copy number of the rRNA sequences ($10^3 – 10^4$ ribosomes/cell) (Bremer and Dennis 1996) and the single

stranded nature of RNA which facilitates probe binding. Cells from an environmental sample are fixed onto a microscope slide, and incubated with the fluorescently labelled probes that enter the cells and hybridize to their specific complementary strands. Non-hybridized probes are washed away and the cells are viewed under an epifluorescent microscope for the identification and quantification of specific genera or species (O'Donnell and Whiteley 1999). Probes specific to acidophilic genera and species have been designed for *Leptospirillum, Acidiphilium, Acidithiobacillus,* and several other AMD common species, making this technique particularly useful in examining AMD communities (Schrenk et al. 1998; Edwards et al. 1999; Bond and Banfield 2001).

A major disadvantage to FISH is the need for prior knowledge of the environment and system, so that the fluorescent probes can be synthesised for specific genera or organisms. The number of probes that can be used in each experiment is also limited, allowing researchers to examine variation of only a subset of the community (Malik et al 2008), a drawback to previous seasonal and AMD community analyses (Schrenk et al. 1998; Edwards et al. 1999; Bond and Banfield 2001).

Recently, target genes, again often rRNAs, have been amplified, cloned, and sequenced to more completely characterize microbial communities. In these techniques, the DNA is extracted from an environmental sample and the marker gene (16S rRNA) is PCR amplified and cloned into a suitable vector. The vectors are then screened for inserts and randomly sequenced to determine an overview of the species present and their relative

abundance. Initial cloning based AMD research done by Bond and colleagues (2000) identified considerable microbial diversity at the Iron Mountain (California, USA) AMD site, leading to much of the present day work done. This method of community characterization is labor intensive and does not provide the amount of community information that direct sequencing obtains with next generation sequencing (explained below).

More recently next generation sequencing methods, targeting marker genes are being used to characterize microbial environments. With the use of next generation sequencing, researchers can obtain billions of environmental sequence reads for a fraction of the previous sequencing costs (Puritz and Toonen 2013). High throughput, next generation sequencing methods are capable of producing huge read counts due to sequencing in parallel. Parallel sequencing refers to the production of micro-scale reactions, in which DNA is attached to separate beads or surfaces where sequencing occurs in parallel. There are several different next generation sequencing techniques, but here I will focus on Roche 454 pyrosequencing due to its common use in ecological research (Puritz and Toonen 2013).

454 pyrosequencing is a high throughput sequencing technique capable of producing over 300,000 bp per hour and read lengths up to 1,000 bp (Freeberg et al. 2012). Direct characterization techniques have been applied to many environmental systems including terrestrial soil environments, deep sea environments, and acid mine drainage (AMD) sites

(Shokralla et al. 2012). In addition to the large volume of data, another advantage of high throughput next generation sequencing techniques is sequencing DNA directly extracted from the environment, without PCR amplification. The lack of amplification allows for relative quantification of microbes based on abundance of reads. Amplification-free next generation sequencing techniques are referred to as direct sequencing and can produce tens to hundreds of thousands of reads (sequences from individual strands of DNA) that can be used for species identification as well as abundances (Lee et al. 2012).

With constant advances in next generate sequencing techniques, not only are read lengths increasing but the cost of sequencing is continually decreasing, allowing for continued and more complete community characterization. The analysis of many environmental communities using next generation sequencing, including pyrosequencing, has allowed a much greater understanding of microbial communities. Additionally, high throughput techniques have shed light on a variety of microorganisms in AMD that are in much lower abundance, termed the rare biosphere (Sogin et al. 2006), many of which were not previously characterized (Auld et al. 2013; Lee et al. 2012; Mohapatra et al. 2011).

1.8 Distinguishing Between DNA from Viable and Non-Viable Cells

One aim of my research is to characterize the variation in the living, microbial community through time. To achieve this aim, it was critical that I could differentiate between DNA from viable and non-viable cells. It has been shown that DNA can persist

in an environment water sample anywhere from several days, to three weeks and potentially longer depending on the environment (Josephson et al. 1993). In my research, I needed to identify the organisms that were active in a community at a specific time point and not confuse them with naked DNA or non-viable organisms. Previous work has shown inhibition of PCR from DNA from non-viable cells from environmental samples through the inclusion of various intercalating agents, including ethidium monoazide (EMA - Waring 1965), and propidium monoazide (PMA - Nocker et al. 2007). Both molecules function similarly and, once photoactivated, produce a reactive nitrene that covalently binds DNA, subsequently inhibiting PCR reactions. EMA, however, was also found to greatly reduce the amount of amplification from viable DNA (Flekna et al. 2007; Nocker et al. 2006). To avoid this reduction, the use of PMA has recently been proposed (Nocker et al. 2006). The use of PMA has been validated in environmental applications in distinguishing viable/non-viable cells (Nocker et al. 2007; Nocker et al. 2007), and as such was used here to better characterize the seasonal AMD environment.

1.9 Objectives

AMD environments have been the focus of considerable research due to their widespread production around the world, large negative environment effect, and huge costs associated with remediation. Although these environments were initially thought to contain simple microbial communities, continued research using modern techniques have

identified a more complex and variable AMD community. The three objectives of my research were to:

1) Investigate, describe and quantify the seasonal variation in the AMD community across Bacterial, Archaeal, and Eukaryote domains.

2) Determine geochemical and physical AMD properties over the seasonal sampling dates.

3) Attempt to understand the correlations between the seasonal AMD geochemical and physical properties and microbial community profiles.

This work should allow a greater understanding of the seasonality in AMD microbial communities and factors which influence its variation.

Chapter 2

2.1 Introduction

Acid mine drainage (AMD), characterized by low pH and extremely high metal (e.g. iron, nickel, copper, and cadmium) and mineral concentrations (e.g. pyrrhotite, and pyrite), is the largest environmental problem facing the mining industry (Egiebor and Oni 2007). It is estimated that 7 billion tons of mine tailings covers about 41,000 hectares of land in Canada, and the estimated cost of remediation is between 2 to 5 billion dollars depending upon the method of treatment (MEND 1994). The production of AMD is largely controlled by microbial oxidation of dissolved iron sulfides tailings from mineral and coal mining processes. Understanding the variation, diversity, and role of AMD microorganisms is crucial to creating new prevention and even bioremediation strategies. Bioremediation being a modern approach using microorganisms to increase the alkalinity and immobilise the metals of AMD sites (Johnson and Hallberg 2005), and an understanding of community variation, biotic and abiotic interactions, and environmental factors are all necessary for successful AMD bioremediation (Hallberg 2010; Boopathy 2000). The central role microbe's play in the production of AMD highlights the importance of both site and seasonal specific microbial community variation and understanding the effect of community structure on AMD production. A greater understanding of community variation could potentially be used for targeted microbe, site, and season specific remediation strategies.

Variation of the seasonal AMD community is not understood, and limited previous work has shown seasonal AMD communities are controlled by physical and geochemical site properties and as such are different between sites (Leduc et al. 2002; Edwards et al. 1999; Schrenk et al. 1998). Previous seasonal characterization work performed has utilized either culture dependant methods (e.g. Leduc et al. 2002) or domain and species specific probes (Edwards et al. 1999; Schrenk et al. 1998), with both methods targeting specific pre identified species. Culture dependant methods are known to suffer from culturing biases (Amann et al. 1995) that make identifying the complete community and seasonal variation difficult. To circumvent culturing bias, many culture independent methods such as fluorescent *in situ* hybridization, denaturing gradient gel electrophoresis, and metagenomics are commonly used to examine community structure but many methods still rely on PCR amplification or time consuming cloning and sequencing which limit the number of samples that can be processed (Hirsch et al. 2010; Mohapatra et al. 2011). Continued advances in next generation sequencing techniques, including reduced cost and increased sequence data production are allowing a more complete AMD community to be examined for both community structure and relative species abundances.

The AMD microbial community was traditionally thought to be simple, consisting of iron oxidizing species (i.e. Leduc et al 2002), mainly *Acidithiobacillus ferrooxidans*, due to the relatively small number of species that could be cultured and the described role of *At. ferrooxidans* in iron oxidation (Schrenk et al. 1998). The production of AMD is limited by iron-oxidizing chemolithotrophs such as *At. ferrooxidans*, specifically in the conversion of ferrous to ferric iron (Singer and Stumm 1970). More recently another iron

oxidizer, *Leptospirillum ferrooxidans* has received considerable attention and has been found to out-compete *At. ferrooxidans* in lower pH conditions, often found in biofilms (Schrenk et al. 1998; Bond et al. 2000). *At. ferrooxidans* and *L. ferrooxidans* are often dominant members of AMD communities, although their abundances have been found to vary greatly depending upon conditions and sites (Edwards et al. 1999; Schrenk et al. 1998; Tan et al. 2009). Recently several other organisms including the Archaeal *Ferroplasma* spp., and several Bacterial species including *Leptospirillum* group III, *Gallionella ferruginea* and *At. ferrovum* have all been found to be dominant organisms in AMD sites, and are suggested to be playing ferrous iron or sulfate oxidation roles (Bond et al. 2000; Hallberg et al. 2006; Tan et al. 2007; Huang et al. 2011). The number of ferrous iron oxidizing prokaryotes is now known to be diverse and further research into the AMD microbial community has also identified an array of neutrophilic, and acidophilic chemolithotrophs as well as heterotrophs (Auld et al. 2013; Hallberg 2010; Johnson 2003; Hallberg and Johnson 2001). Additionally, with culture independent, and next generation sequencing techniques the known AMD microbial diversity is increasing, including neutrophilic organisms originally not thought to survive in AMD such as *Legionella* (Hao et al. 2010; Auld et al. 2013), to rare abundance genera such as *Granulicella*, *Acidocella*, and *Alcyclobacillus* species (Auld et al. 2013). Overall, these studies suggest a more complex and dynamic community than originally thought, warranting further characterization and research.

Here we characterize the prokaryote and Eukaryote AMD community, including seasonal variation, from Vale's central tailings facility in Copper Cliff, ON, Canada using direct

pyrosequencing. Our characterization of the prokaryote and Eukaryote microbial communities across three winter and three summer sampling times shows significant seasonal community variation. This seasonal community variation suggests that AMD production varies between seasons, along with species abundances. Correlations between water chemistry and specific taxa within the community suggest species abundances are directly related to geochemical and physical AMD properties. The identified AMD community, geochemical, and physical property variation likely play a large effect on AMD production and suggests that future work and AMD remediation strategies should take into account seasonal community dynamics.

2.2 Methods

2.2.1 AMD Sample Collection & Physicochemical Analysis

Acid mine drainage (AMD) water samples were collected from Vale's central tailings pond in Copper Cliff Ontario in 2012 for direct sequencing and chemical analysis. Water samples were collected at three winter and three summer dates to determine inter-, and intra-, seasonal variation in the AMD microbial community. Within a season, collections were approximately two weeks apart. The winter dates were: Feb 22nd (W1), Mar 1st (W2), and Mar 14th (W3). The summer dates were: July 19th (S1), Aug 2nd (S2), and Aug 21st (S3). To better assess the overall AMD pond microbial community (and not simply the diversity at one location in the pond), water was collected from three pond locations and pooled (A, B, and C – Figure 1); two locations were several feet off the shoreline and

approximately 75 meters apart (UTM: 17T 0493925m E, 5146461m N – Location A, and UTM: 17T 0493953m E, 5146510m N – Location B) and one location was more central within the pond (UTM: 17T 0493932m E, 5146486m N – Location C). Winter samples were taken by drilling holes through the ice, being careful not to disturb the sediment. Summer samples were taken from the shore for the two shallow samples, and using hip waders and a collection pole; again care was taken not to disturb the sediment. For each collection time point, three liters of AMD were sampled from each of the three different pond locations, A, B and C using sterile 1L bottles; nine 1L bottles in total on each sampling date. Equal portions from pond locations A, B, and C were pooled totaling three (triplicate), two liter samples, which cells and DNA extractions were performed on.

Figure 1: AMD sampling site, Copper Cliff, Ontario Canada; three pond locations were used for drainage sampling labelled A-C.

2.2.2 AMD Geochemistry Analysis

An additional 1L of pooled AMD was collected to determine the AMD geochemical properties. Samples were analysed by TestMark laboratory for total metals by inductively coupled plasma mass spectrometry anions by ion chromatography, and

Metrohm meters were used for pH, alkalinity, and conductivity. Water and air temperature, ice thickness, and cumulative snow were recorded on site.

2.2.3 DNA Extraction, PMA Treatment, & Pyrosequencing

Microbial cells were filtered from the triplicate, two liter, pooled AMD samples using a 0.2 um membrane filter. One membrane filter was used per 1L of AMD for more efficient cell filtration (i.e. two filters were required per replicate). Cells were collected from the membrane filters by first submersing the filters in 7mL of sterile AMD and lightly vortexing the filters and AMD solution until the majority of precipitate was seen to be removed from the filters. Propidium monoazide (PMA) treatment was used to prevent sequencing of DNA from dead cells (Nocker et al. 2006). PMA was added to each 7mL cell sample to a concentration of 50uM, followed by 5 min incubation in the dark, and 7 minute incubation under a 500 watt light source with constant shaking. Samples were then centrifuged for 10 minutes at 5,000 rpm to pellet the cells and the supernatant discarded. The pellet was then re-suspended in 500uL of sterile water and DNA immediately extracted using a MO BIO Power Water DNA isolation Kit, following the MO BIO manufacturer protocol (MO BIO Laboratories, Inc. California, USA). Extracted DNA from six sampling dates (three winter and three summer) was sent to Research and Testing Laboratories (Texas, USA) for Bacterial, Eukaryote, and Archaeal sequencing on a Roche 454 FLX/FLX + pyrosequencing platform. The 16S rRNA gene was sequenced from prokaryotes using the 28F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3') universal Bacterial primers targeting the

V1 –V3 regions, and from Archaea using the Arch349F (5'-GYGCASCAGKCGMGAAW-3') and Arch806R (5'- GGACTACVSGGGTATCTAAT-3') universal Archaeal primers targeting the V3 – V4 regions. The 18S rRNA gene of Eukaryotes was sequenced using the universal Euk7F (5'-AACCTGGTTGATCCTGCCAGT-3') and Euk570R (5'-GCTATTGGAGCTGGAATTAC-3') primers targeting the V1 – V3 regions.

2.2.4 Processing of Pyrosequencing Data

Sequence data was processed using publically available software to identify our seasonal microbial community profiles. Bacterial and Archaeal 16S rRNA sequences were quality filtered using Qiime 1.5.0 (Caporaso et al. 2010) set to default parameters, removing sequence lengths below 200bp, above 1000bp or those that did not meet a minimum quality score of 25 and sequence data was denoised using Qiime default parameters. The complete sets of filtered sequences were classified using the Ribosomal Database Project –II classifier (RDP-II). Classification was performed to the class and genus levels using an 80% threshold (Wang et al. 2007). The Eukaryote 18S rRNA sequences were also processed using Qiime using quality filtering identical to that of the prokaryote data and Qiime default parameters. Eukaryote sequences were classified to phyla and class using Qiime, since the RDP-II pipeline does not support 18S processing. OTUs were chosen against the Silva 108 release (Quast et al. 2013) and clustering performed at 97% sequence identity. The resulting OTUs were then classified against the Silva 108 dataset release.

2.2.5 Inferring Phylogeny

We performed phylogenetic analyses of the dominant taxa from Bacteria, Eukaryotes, and Archaea. Representative prokaryotic OTU sequences were selected using the RDP pipeline as follows. Qiime filtered sequencing data was aligned and complete linkage clustering performed using RDP pipeline with a maximum distance of 15%. Representative sequences were then chosen from the clusters using a maximum distance of 3%. OTU's were checked for chimeric sequences using ChimeraSlayer (Haas et al. 2011) and dominant representatives (> 1% sequence counts) for each sampling date were used in the phylogenetic analysis along with the two closest nBLAST (Altschul et al. 1990) hits. nBLAST searches were performed using cultured databases first and those sequences that showed no matches were then BLAST against environmental uncultured organisms. Phylogenetic analysis was performed using maximum likelihood methods and Tamura-Nei model with 1000 bootstrap replicate implemented in Molecular Evolutionary Genetics Analysis (MEGA 5) (Tamura et al. 2011). Similarly, dominant Eukaryote sequences (>1% sequence reads) were chosen from the complete OTU table, in this case using Qiime not the RDP pipeline, and these representative sequences used to build the Eukaryote phylogeny identical to the prokaryote dataset.

2.2.6 Statistical Comparison of Communities

Alpha (within community) and beta (between communities) diversity analysis for both prokaryote and Eukaryote datasets were performed using Qiime. The Bacterial, Archaeal, and Eukaryote community sequencing datasets were quality filtered and clustered at the 97% sequence identity within each domain. Representative sequences for all three domains were chosen and aligned using the default PyNAST parameter and representative sequences were aligned using the core Silva 108 aligned set implemented in Qiime. Alignments were filtered using default parameters and phylogenies for statistical analyses were built using fasttree, all implemented in Qiime, in order to perform beta analyses. Principle coordinate analysis, Unifrac distances, Unifrac significance, and P-tests were performed on rarefied (equal sampling depths) datasets to compare within and between seasons. Chao1 (Chao 1987) and Shannon (Shannon and Weaver 1949) indices were calculated on rarefied data using Qiime to determine the species richness and evenness of distribution.

2.2.7 PMA Analysis

An additional two samples were taken during the final summer sampling time point (S3) to assess the effect of PMA treatment on viable/non-viable cells. Both additional samples were collected and analysed using the same procedures explained above, except one sample was treated with PMA and the second sample was not PMA treated prior to 454 pyrosequencing. Extracted DNA was sequenced using only the universal Bacterial primers. The obtained sequence data was processed and identical statistical tests performed as on seasonal community sequence data.

2.2.8 Chemical Statistical Analysis

To correlate physical and chemical properties across the sample dates to changes in the AMD community, multivariate correlation analysis (Redundancy Analysis – RDA) was performed with Canoco 4.0 for windows (Ithaca, New York, USA) and RDA triplots were created. Additionally to correlate taxonomic groups with AMD chemical and physical properties, Spearman's rank correlations were calculated with JMP version 10 (SAS - Cary, North Caroline, US). All multivariate analyses were performed on all genera with presence at more than two seasonal points.

2.3 Results

2.3.1 Chemical Analysis

Geochemical and physical AMD properties were determined for each sampling time and compared across samples to identify possible trends or variation in AMD properties across sampling dates. The pH, temperature, conductivity, and concentration of several key AMD metals, are plotted across the sampling times in Figure 2. Metal concentrations, pH, and conductivity all decrease over the course of the winter. In contrast, metal concentrations are fairly constant during the summer, showing only a slight increasing trend. The pH and conductivity remain relatively constant in the summer, although the pH is lower in summer than in winter. Average yearly, winter, and

summer metal concentrations are shown in Table 1 and the complete quantified list of AMD properties for the six sampling dates is shown in Supplemental Table 1.

Table 1 Average yearly and seasonal AMD metal concentrations from key AMD related metals, including standard error. All measurements in mg/L.

	Yearly	**Winter**	**Summer**
Sulphur	724 ± 89.5	689 ± 194	760 ± 32.2
Sulphate	2,675 ± 291	2,363 ± 569	2987 ± 59.0
Iron	293 ± 78.5	330 ± 170	256 ± 30.7
Nickel	30.4 ± 4.86	31.8 ± 10.6	29 ± 2.19
Copper	1.53 ± 0.23	1.82 ± 0.35	1.24 ± 0.26

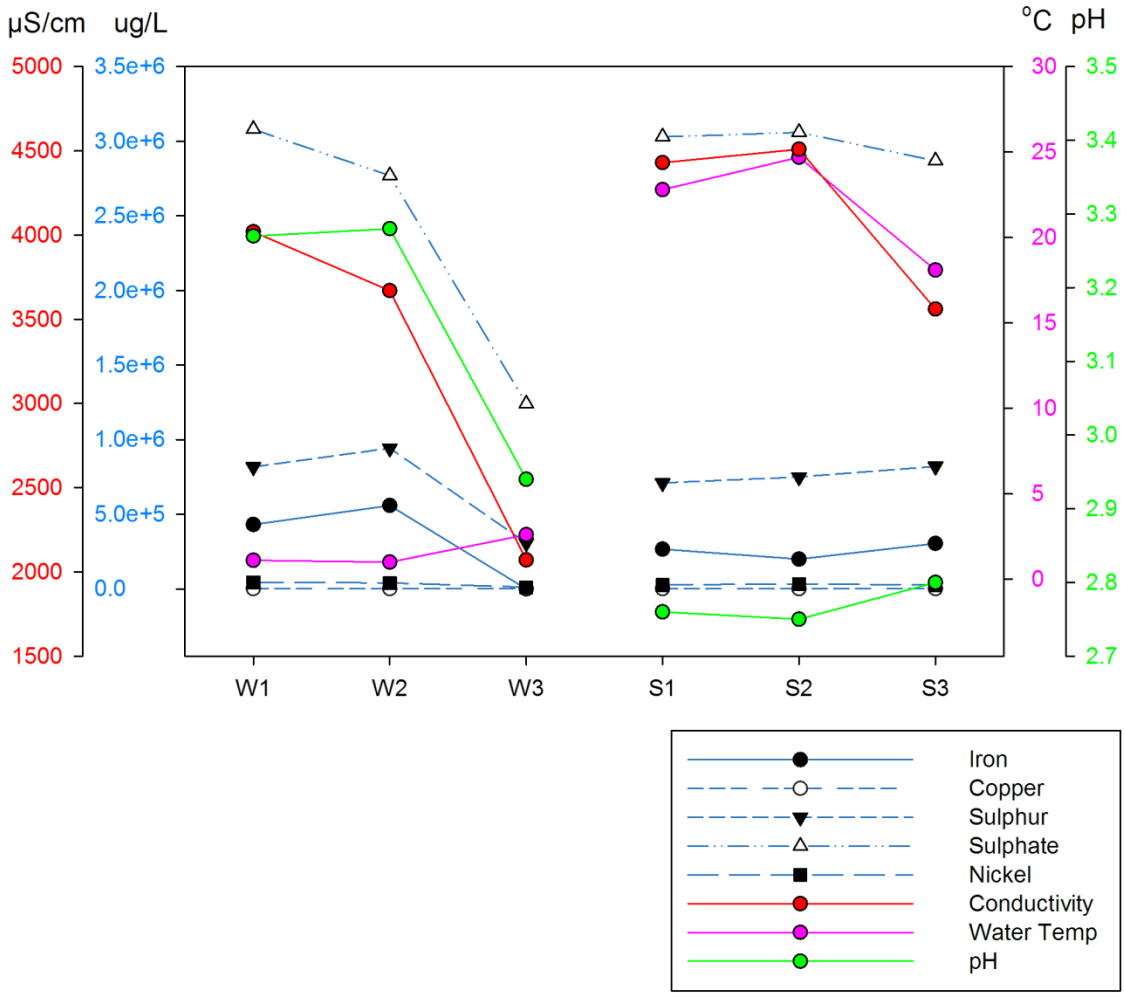

Figure 2: AMD-relevant metal and physical properties from the six seasonal sampling dates. Colored tramlines correspond to their identical colored axes.

2.3.2 rRNA Pyrosequencing Analysis

Bacterial, Archaeal, and Eukaryote small subunit rRNA gene sequences (16S, 16S and 18S, respectively) were directly sequenced from the water samples. Direct sequencing data was obtained for the Bacterial and Eukaryote domains for all six sampling dates

(Figure 3). Over 525,000 trimmed and quality filtered gene sequence reads were analyzed to assess the microbial diversity of the AMD site over the six sampling dates (Table 2). Concentration of DNA extracted from each seasonal sample was not normalized making direct correlations or inferences on actual numbers of community members impossible, although drainage samples were all extracted using identical processes and thus only community proportions and general trends can be observed. Overall the Bacterial read abundance was relatively constant, with a slight increasing trend, over the winter months and decreased trend over the summer from an initial high. Eukaryote sequence abundances were variable over the winter months, and had a slight decreasing trend over the summer. In contrast to Bacteria and eukarytoes, we were only able to obtain sequence data from Archaea from the winter samples and the last summer sample (S3). The number of Archaeal sequences was also much more variable than in the Bacterial and Eukaryote domains, increasing almost 21 fold from first to last winter samples. We recovered no Archaeal sequence reads at all from S1 and S2 and very few reads from S3 (405). Our inability to obtain Archaeal sequences from S1 and S2 presumably reflects the very low abundance, or absence, of Archaeal DNA in these two summer dates; and we were unable to amplify DNA using small subunit rRNA PCR primers specific to Archaea.

Table 2 Number of classified small subunit rRNA sequences to Bacterial, Eukaryote, and Archaeal domains along with average sequencing length over the six sampling times

	Bacteria		**Eukarya**		**Archaea**	
	Average Read Length	Bacterial Reads	Average Read Length	Eukaryote Reads	Average Read Length	Archaeal Reads
W1	343pb	46,062	309pb	31,628	377pb	935
W2	340pb	55,437	330pb	41,939	377pb	1,733
W3	359pb	56,004	354pb	19,289	371pb	19,589
S1	422pb	78,780	338bp	37,219	--	--
S2	432pb	43,274	358pb	31,073	--	--
S3	414pb	37,821	384pb	27,116	380pb	405

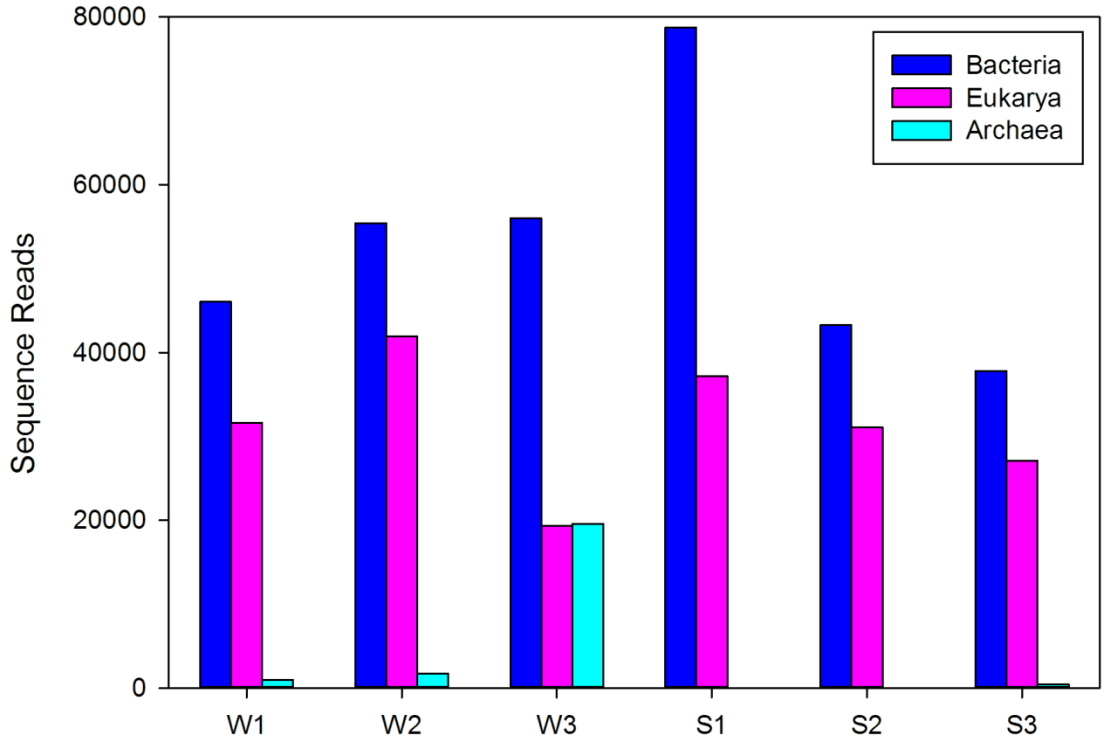

Figure 3: Graphical representation of total domain targeted sequences for prokaryotes and Eukaryotes during each winter and summer sampling date.

2.3.3 Acid Mine Drainage Microbial Community Profile

Bacterial Community

The AMD microbial community composition varied both within and between seasons. Comparison of the AMD community was performed using Unifrac's phylogenetic based metric, (Lozupone and Knight 2005) implemented in Qiime. Unifrac based principle coordinate analysis (PCoA) represents community differences in three dimensional space and can be used to identify similar or dissimilar communities. We generated plots based on seasonal community change and species abundance in which each point on the graph

represents the community profile (three points per sample date). The Bacterial PCoA plot shows strong summer and winter seasonal community clustering with tighter summer than winter clustering suggesting a more similar and constant summer Bacterial AMD community (Figure 4). For each Bacterial paired datasets a Unifrac phylogenetic based distance metric was calculated and examined in addition to PCoA clustering trends. Small Unifrac distance indices indicate a more similar community, sharing similar phylogenetic lineages and evolutionary history, while larger values indicate more distinct communities (Costello et al. 2009). Unifrac distances can either be weighted, calculated including species abundance, or unweighted, with only presence/absence (without including abundance). Weighted distances give an idea of evolutionary history and population evenness, while unweighted distances only assess the evolutionary history and shared lineages (Lozupone et al. 2007). In our data sets, both weighted and unweighted Bacterial Unifrac distances show similar trends (Figure 5). Not surprisingly, the largest Unifrac distance is between winter and summer Bacterial communities, indicating high seasonal community variation. The within-winter Bacterial Unifrac distance was higher than the summer Bacterial distance, indicating more winter community variation than within summer (Figure 5). In order to determine if seasonal communities differ statistically, Unifrac's phylogenetic based significance test and P tests were performed – Figure 5 (Lozupone and Knight 2005). The P test implemented in Unifrac takes into account the presence or absence of lineages between communities, where as Unifrac's significance testing utilizes lineages as well as sequence similarity or evolutionary distance (Lozupone and Knight 2005). Bacterial Unifrac significant and Unifrac P tests

indicate that Bacterial communities are all significantly (<0.001) different, both between and within seasons.

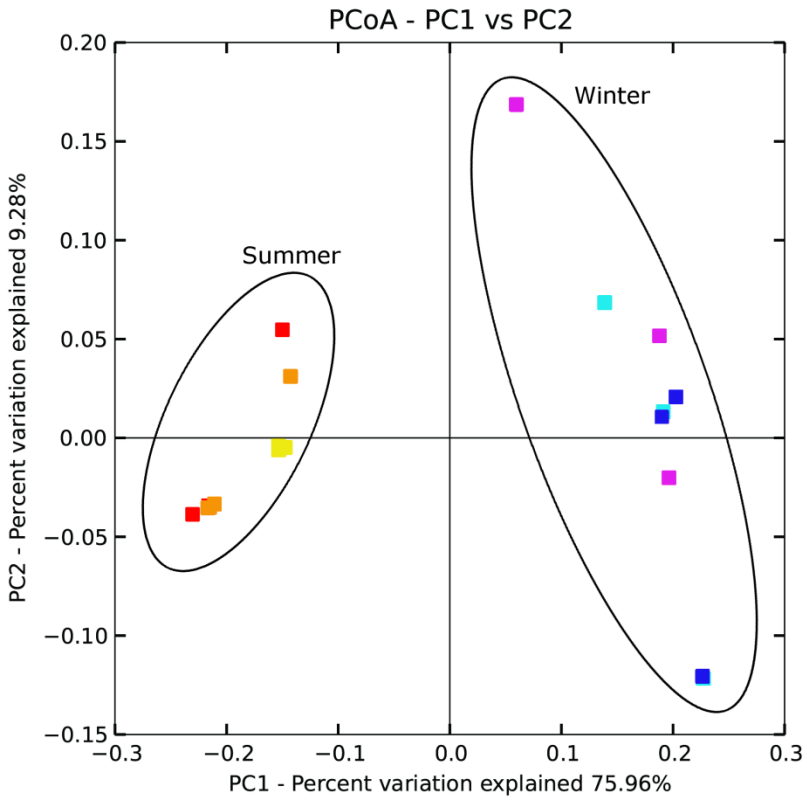

Figure 4: Bacterial weighted Unifrac PCoA plot depicting seasonal community similarities. Triplicate sampling communities are represented; W1 (Blue), W2 (Light blue), and W3 (Purple) represents the first, second, and third winter sampling dates, and S1 (Red), S2 (Orange), and S3 (Yellow), the first, second, and third summer dates respectively.

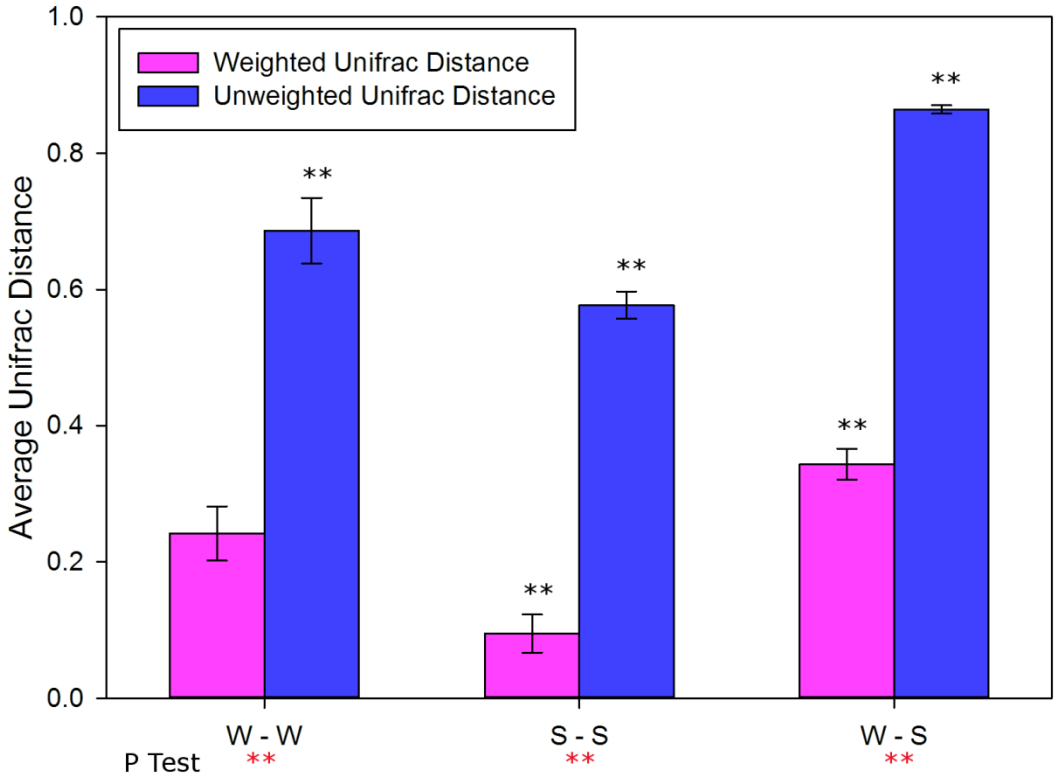

Figure 5: Mean Bacterial environmental distance metrics calculated between each pair of seasonal environments with standard error. W-W, S-S, and W-S represent winter to winter, summer to summer, and winter to summer community comparison distances respectively. Small distance values indicate more similar communities while values closer to one indicate more community variation. Asterisk above the bars indicate weighted or un-weighted Unifrac significance testing and red asterisks represent Unifrac P-test values (* 0.01-0.05, ** indicate <0.001).

Across all samples, the majority of the Bacterial AMD community is comprised of three phylogenetic classes, *Betaproteobacteria*, *Gammaproteobacteria*, and *Alphaproteobacteria*, although the relative contribution of each class varies across samples and especially by season (Figure 6A). Variation in the Bacterial community, apparent in our phylogenetic representation of the dominant Bacterial OTUs (>1% sequence reads, Figure 7) highlights community change between summer and winter

seasons. The Bacterial summer community is largely composed of *Alphaproteobacteria* and *Gammaproteobacteria*. S1 and S2 communities are very similar, with *Alphaproteobacteria* making up 88% and 85% relative abundance and *Gammaproteobacteria* at 9% and 10% abundance respectively. The final summer community (S3) was significantly different from those of S1 and S2 (Unifrac P test and Unifrac significance test <0.001), with *Alphaproteobacteria* and *Gammaroteobacteria* comprising 48% and 47% relative abundance respectively. During the winter months, *Betaproteobacteria* and *Gammaproteobacteria* dominate the AMD, but follow different abundance trends. *Betaproteobacteria* decrease throughout winter from 52% relative abundance in W1 to 6% in W3. Conversely, the *Gammaproteobacteria* initially represent 14% of the winter classified reads but increase to nearly 62% in our last winter sample (W3). During the winter months, the population of *Alphaproteobacteria* remain relatively constant at 1-4% relative abundance.

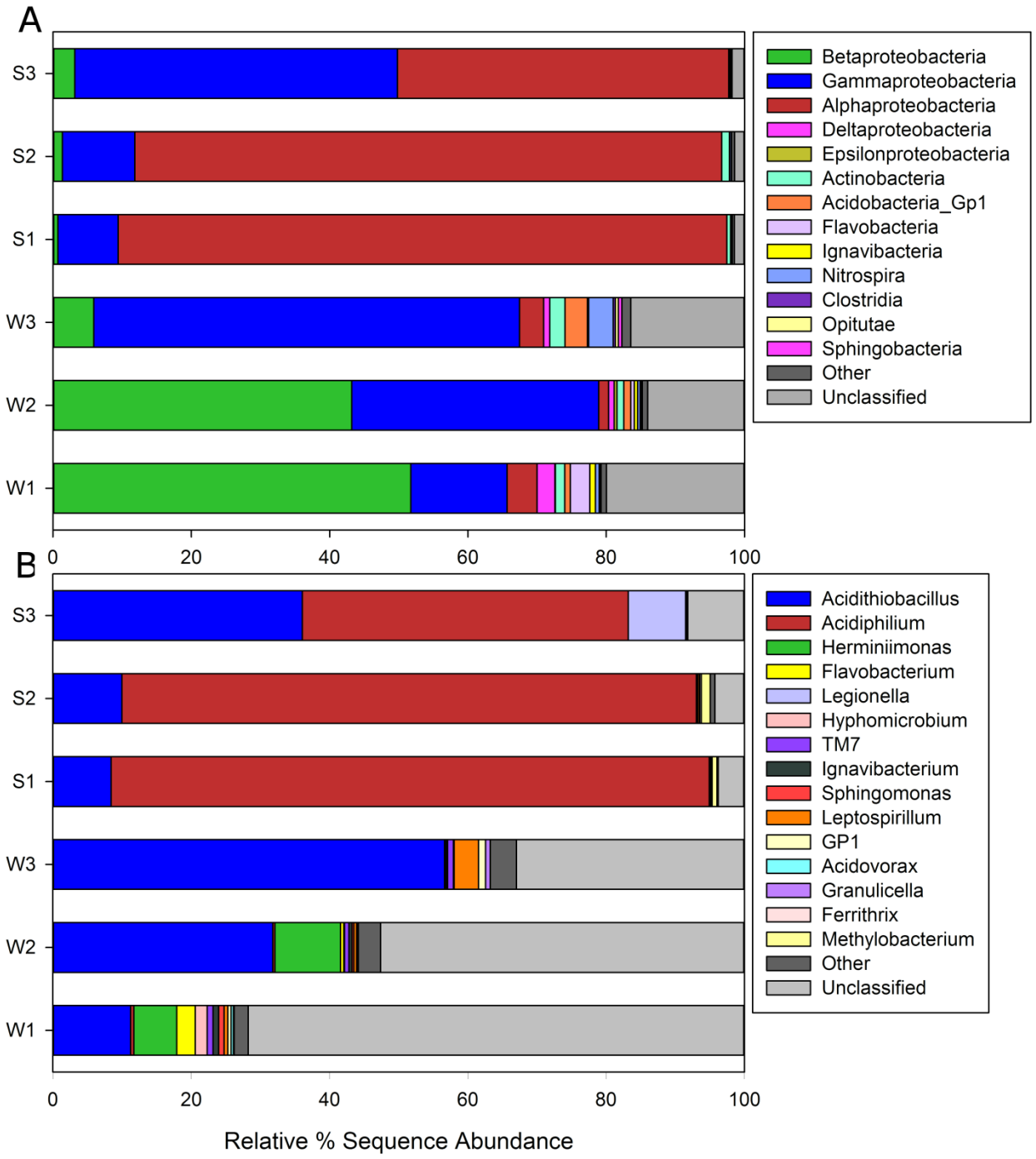

Figure 6: Seasonal Bacterial AMD community profile depicted by relative percent sequence abundance at class (A) and genus (B) levels. Bars W1 – W3 and S1 – S3 represent the community profiles for the first to third winter and summer sampling dates, each date comprising of triplicate pyrosequencing analyses.

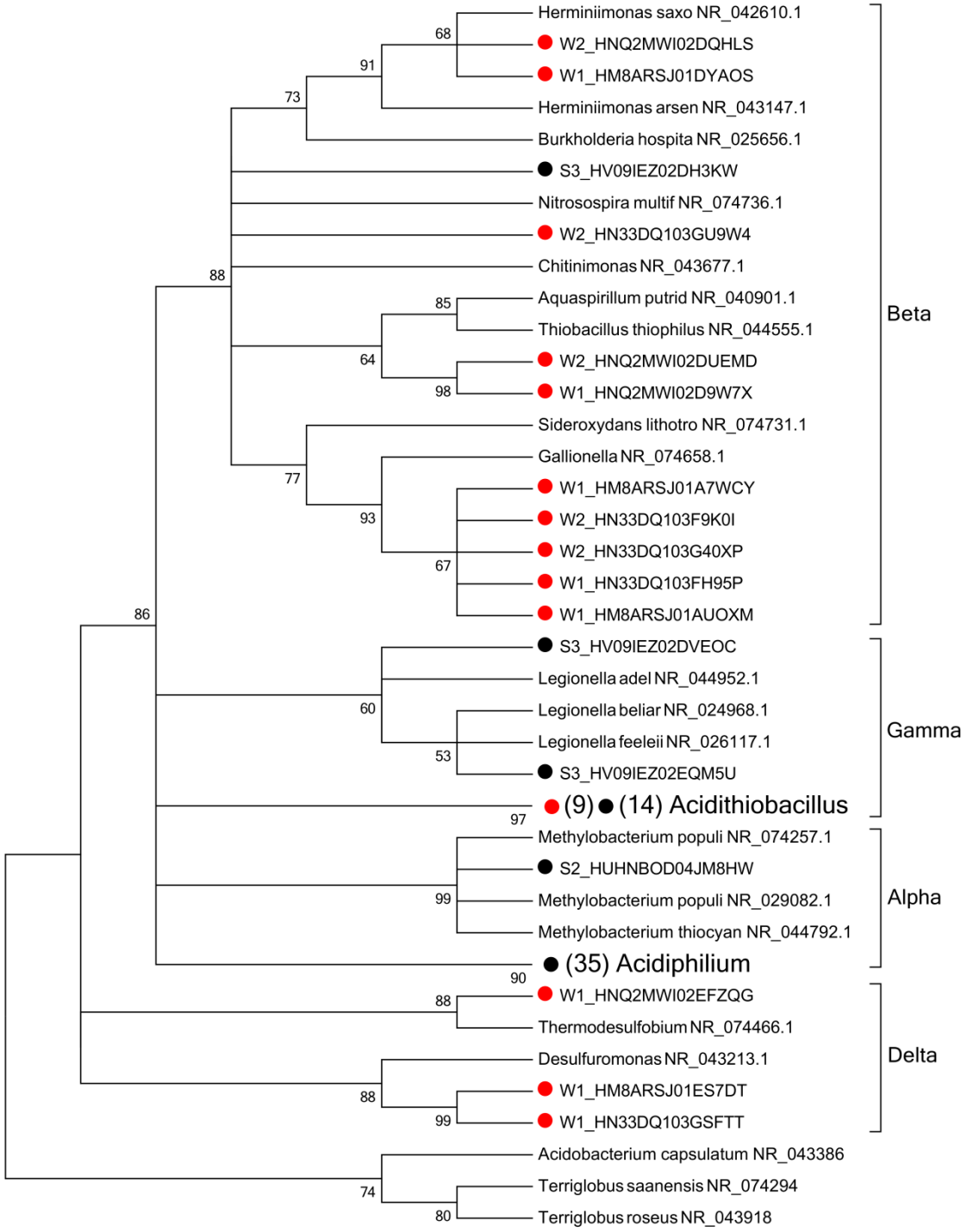

Figure 7: Bacterial maximum likelihood tree representing dominant (>1%) OTU's and their closest BLAST hits. Red and black symbols indicate winter and summer sequencing OTU's respectively, and bracketed values represent OTU abundances along condensed monophyletic groups.

The vast majority, >90%, of Bacterial 16S sequences from the summer samples was confidently classified to genus (Figure 6B) and, overall, the summer Bacterial diversity was found to be relatively low compared to winter. The majority of summer Bacterial sequences belonged to one of two genera, *Acidithiobacillus* or *Acidiphilium*, although the relative abundance of each genus varied across the samples. During the summer, *Acidiphilium* is the most abundant genus, ranging from 87% to 47% relative abundance between S1 and S3 sampling dates. *Acidithiobacillus* is also abundant in summer samples, ranging from 8% in S1 to over 36% in S3. *Legionella* sequences were also found within all sequencing dates but only at >1% total sequences during the final S3 summer date (~8%).

Classification of winter Bacterial sequences to genus was more problematic than summer classification; winter genus classification ranged from 28% in W1, to 47% in W2, to 63% in W3, while all sequences were classified to phyla. The 27% classified W1 sequences included, *Acidithiobacillus* (11% of total sequences), *Herminiimonas* (6%), *Flavobacterium* (3%), Hyphomicrobium (2%) and several other low abundance (less than 1%) genera. The winter samples, especially W1, were more diverse than summer. There were a number of genera that were only found in winter samples, including *Flavobacterium*, *Hyphomicrobium, Sphingomonas*, *Ignavibacterium, Sulphuricella*, and *Sulphuritalea* (Figure 6B). We did not find these specific taxa in the summer. The largest winter community shift was the increase of *Acidithiobacillus* from 11% in W1 to

32% and 57% in W2 and W3. The genus *Leptospirillum*, commonly found in AMD systems (Schrenk et al. 1998; Bond et al. 2000), was also found throughout the year but only in relatively low abundance ranging from a high of 3.5% in W3 to less than 1% in all other, winter and summer, samples.

PMA treatment was not found to statistically alter the Bacterial community profile in comparison to non PMA treated DNA (Supplemental Figure 1A). While the use of PMA did seem to decrease the number of reads obtained from each dominant Bacterial Order (Supplemental Figure 1B), the decrease in read abundance did not have any statistical effects.

Archaeal Community

Across all samples, Archaeal abundance and biodiversity were low compared to the other two domains (Figure 3 & 8). All classified sequences were from only two classes, *Thermoprotei* and *Thermoplasmata*, but a large percentage of sequence data could not be classified (25-56% at class and 70-94% at genus levels). The seasonal Archaeal profile remained relatively constant, but the number of sequence reads obtained throughout the year changed dramatically with the highest number of reads obtained from W3 (19,589) and no sequencing data was obtained from S1 and S2 dates (Table 2). Even with this relative low abundance and diversity the seasons are easily resolved in the PCoA plot (Figure 9). Unifrac weighted and unweighted distances matrices were greater between seasons than within winter, indicating that though less variable, the seasons were distinct.

Unweighted Unifrac significance testing, as well as by phylogenetic P-tests, indicate that the winter and summer Archaeal community were significantly different (<0.001), but no significant variation was detected across the winter samples (Figure 10).

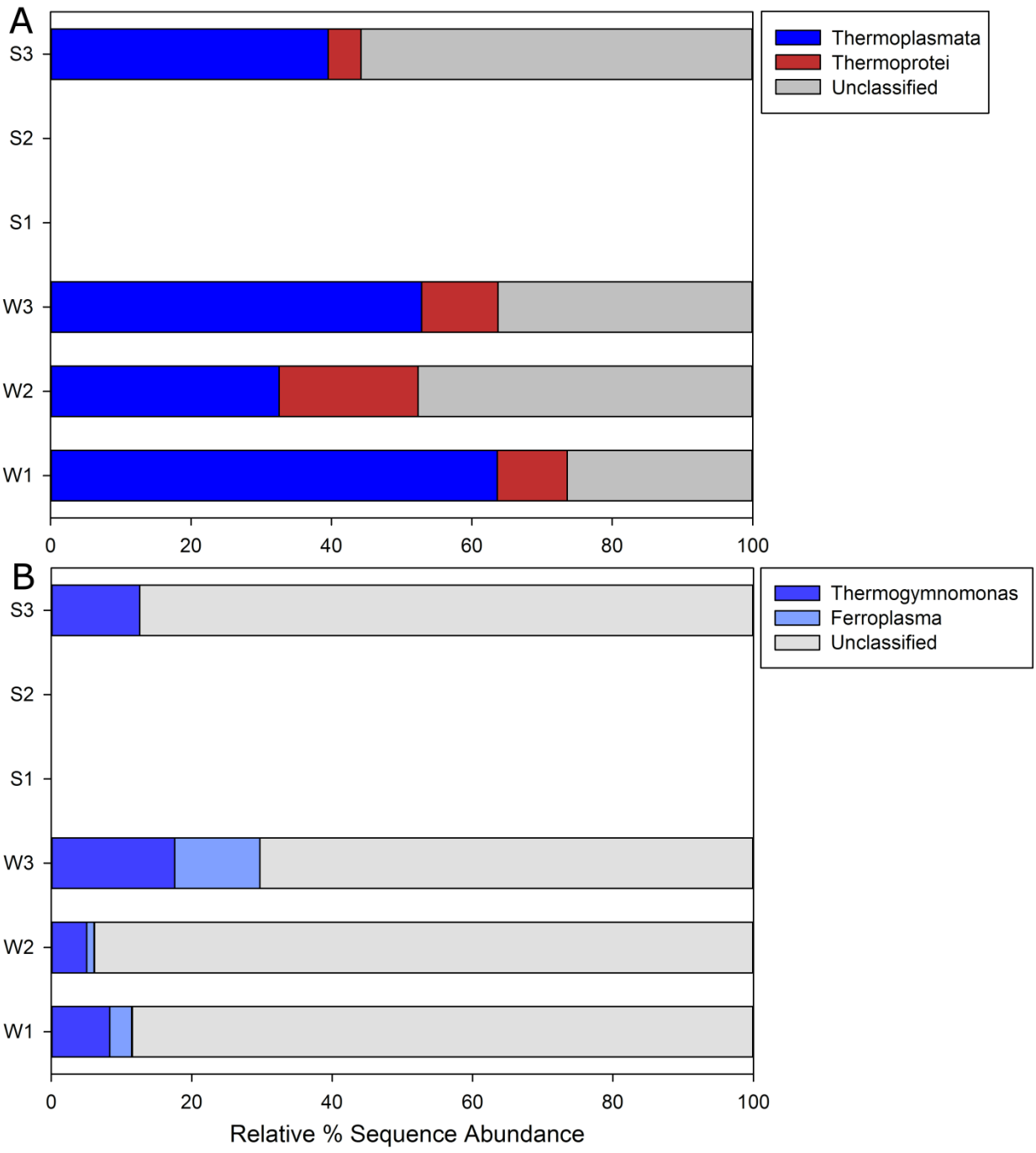

Figure 8: Seasonal Archaeal AMD community profile depicted by relative percent sequence abundance at class (A) and genus (B) levels; no pyrosequencing data was obtained for S1 and S2 Archaeal communities.

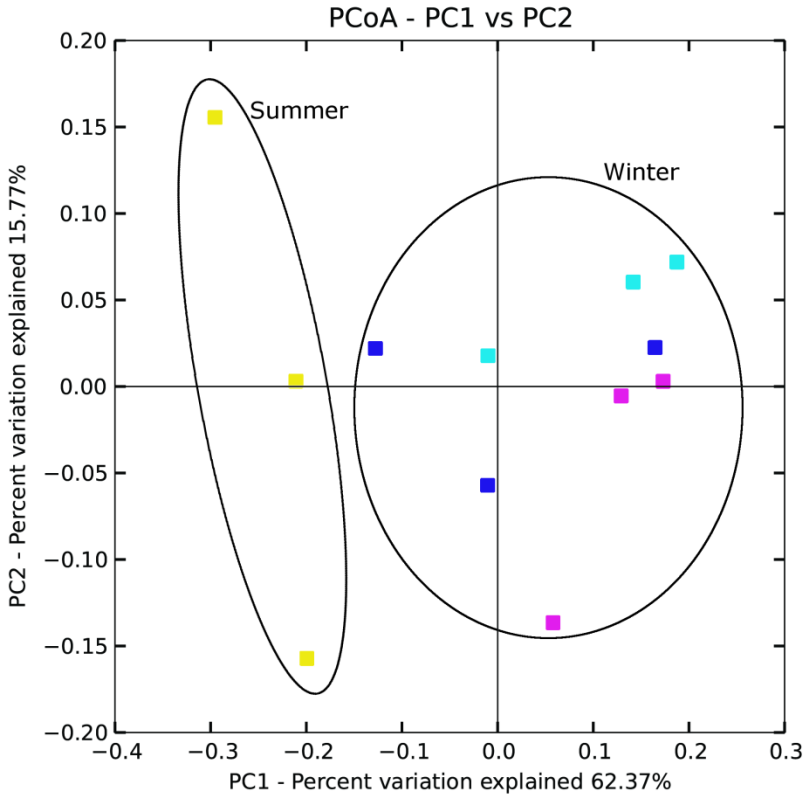

Figure 9: Archaeal Unifrac weighted PCoA plot depicting seasonal community similarities. Triplicate sampling communities are represented; W1 (Blue), W2 (Light blue), and W3 (Purple) represents the first, second, and third winter sampling dates. S3 (Yellow) represents the third summer sampling date; the first two summer dates produced no sequence reads for Archaeans.

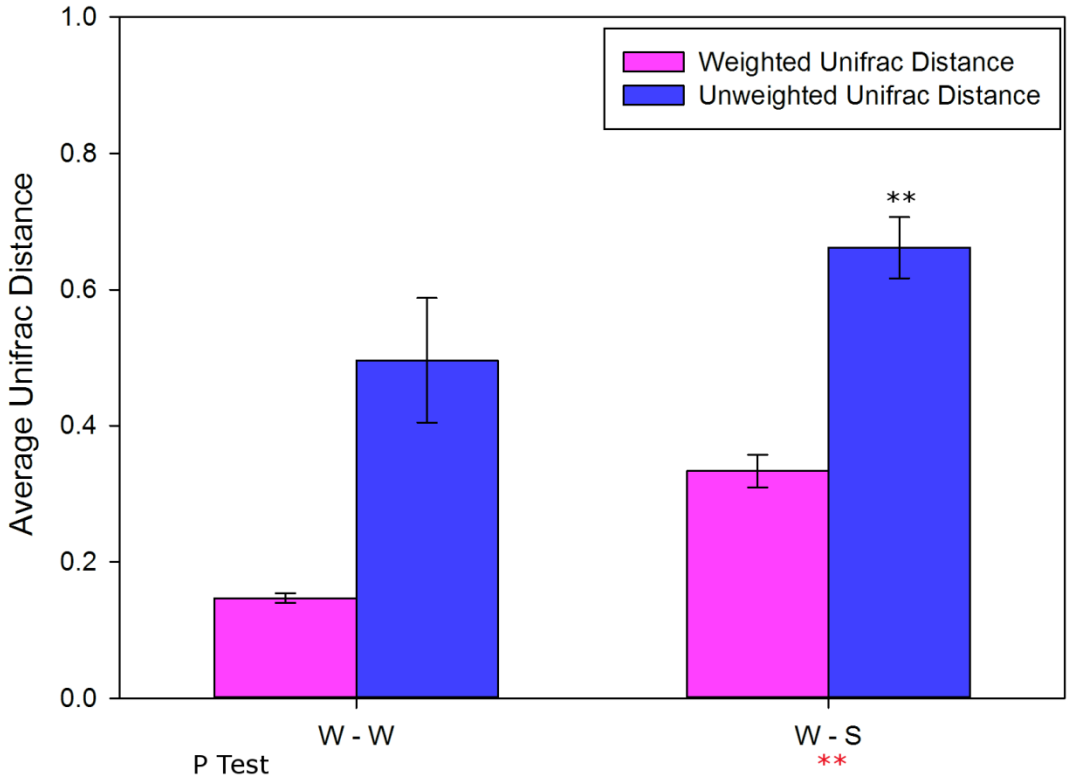

Figure 10: Plotted mean Archaeal environmental distance metrics, distances calculated between each pair of seasonal environments, error bars represent standard error. W-W and W-S represent winter to winter and winter to summer community comparisons distances respectively. Small distance values indicate more similar communities while values closer to one indicate more community variation. Asterisk above the bars indicate weighted or un-weighted Unifrac significance testing and red asterisks represent Unifrac P-test values (* 0.01-0.05, ** indicate <0.001).

A large portion of the Archaeal sequencing data (26 – 56%) while classified to the Archaeal domain, could not be confidently classified to phylum (not shown) or class (Figure 8A) and the majority could not be confidently classified to genus (70 - 94%) (Figure 8B), highlighting our lack of knowledge of AMD Archaea and Archaea in general. The sequences that could be classified to class suggest that the Archaeal community is largely composed of *Thermoplasmata* and *Thermoprotei* with 32 - 60% and

4 - 20% relative abundances respectively. *Thermogymnomonas* and *Ferroplasma* were the only genera confidently identified, and both were found in highest abundance in W3 at 17% and 12%, respectively (Figure 8B). The genus *Thermoplasma* was also identified during the winter but in extremely low percentages (<0.1% abundance).

Eukaryote Community

The Eukaryote microbial community was found to represent a large portion of the AMD microbial community. Eukaryotes were found in lower abundance and diversity than Bacteria, but much higher diversity and abundance than Archaea. The eukaryotic community varied significantly across seasons and tight seasonal clustering is apparent in the PCoA plot (Figure 11). The Eukaryote community contained significant phylogenetic clade length variation (evolutionary difference) and lineage specific variation between seasons (Unweighted Unifrac Significance <0.001, and P Test <0.001) as well as lineage specific variation within summer and within winter seasons (P Test <0.05). Weighted and unweighted Unifrac significance tests indicated no significant differences across same-season samples. Weighted and unweighted distance tests were somewhat contradictory with unweighted distances identify more seasonal community variation than within season, while weighted distances indicate more similar summer than winter or seasonal community variation (Figure 12).

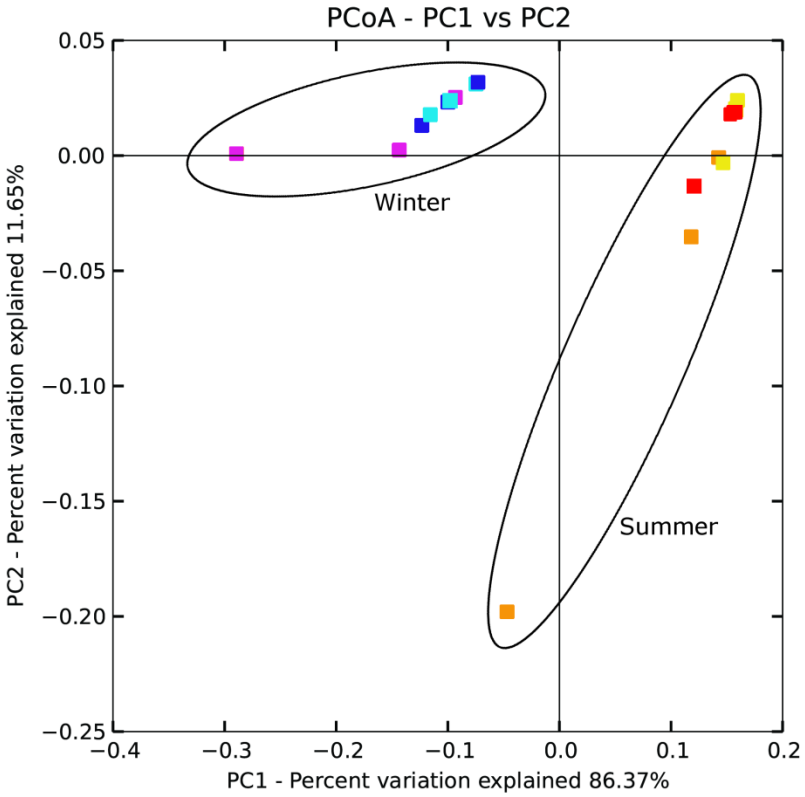

Figure 11: Eukaryote Unifrac weighted PCoA plot depicting seasonal community similarities. Triplicate sampling communities are represented; W1 (Blue), W2 (Light blue), and W3 (Purple) represents the first, second, and third winter sampling dates, and S1 (Red), S2 (Orange), and S3 (Yellow), the first, second, and third summer dates respectively.

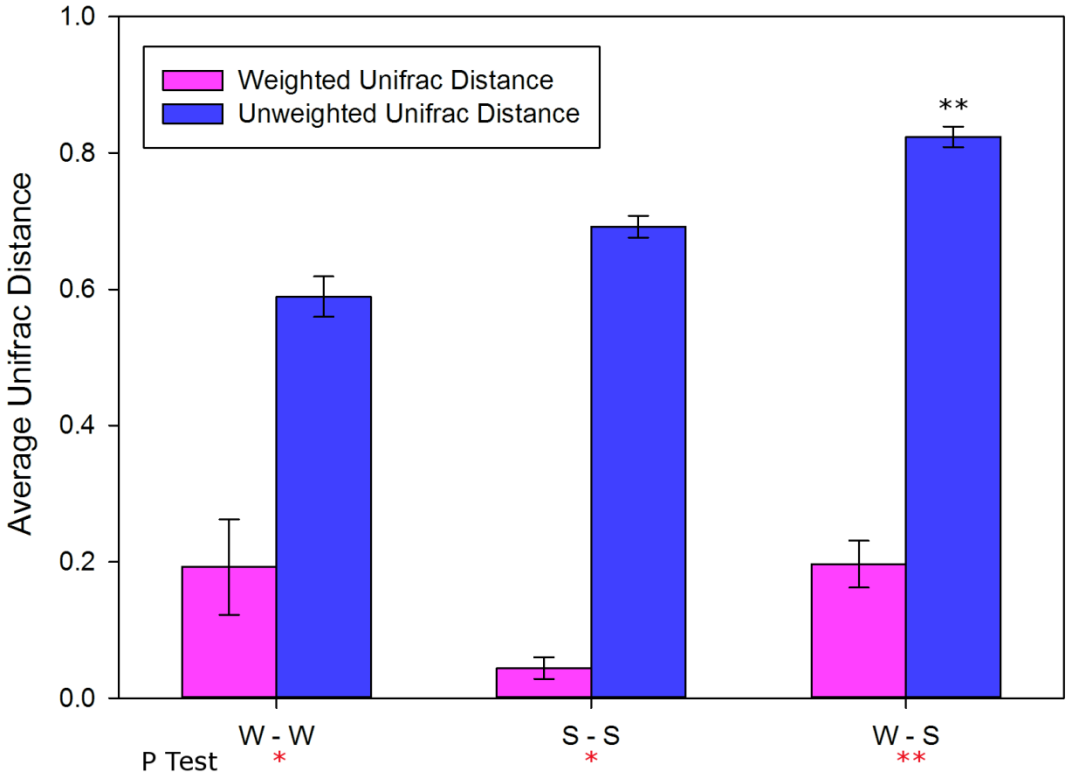

Figure 12: Plotted mean Eukaryote environmental distance metrics, distances calculated between each pair of seasonal environments, error bars represent standard error. W-W, S-S, and W-S represent winter to winter, summer to summer, and winter to summer community comparisons distances respectively. Small distance values indicate more similar communities while values closer to one indicate more community variation. Asterisk above the bars indicate weighted or un-weighted Unifrac significance testing and red asterisks represent Unifrac P-test values (* <0.05, ** <0.001).

Interestingly, our ability to identify eukaryotic sequences, even to phyla, varied enormously between winter and summer samples. The majority of the Eukaryote sequence data from the summer samples while classified to the Eukaryote kingdom could not be classified to the phylum (79 – 98% - data not shown) or to class (78% - 98% - Figure 13). Of the small portion of summer classified Eukaryote sequences, *Chrysophyceae* and *Chlorophyceae* classes of green algae made up the majority, up to

5% and 11% abundance respectively. In contrast, the vast majority of the winter Eukaryote sequences were classified to phylum (>99%) and class (94-99%) levels. The large and diverse phyla, *Stramenophiles* were in highest abundance in W1 and W2 (84 - 91%) largely comprised of the class *Synurophyceae* (89 – 83%). The large fungal phylum, *Basidiomycota* dominated W3 at 93%, composed mainly of the class *Agaricostilbomycetes* (92%).

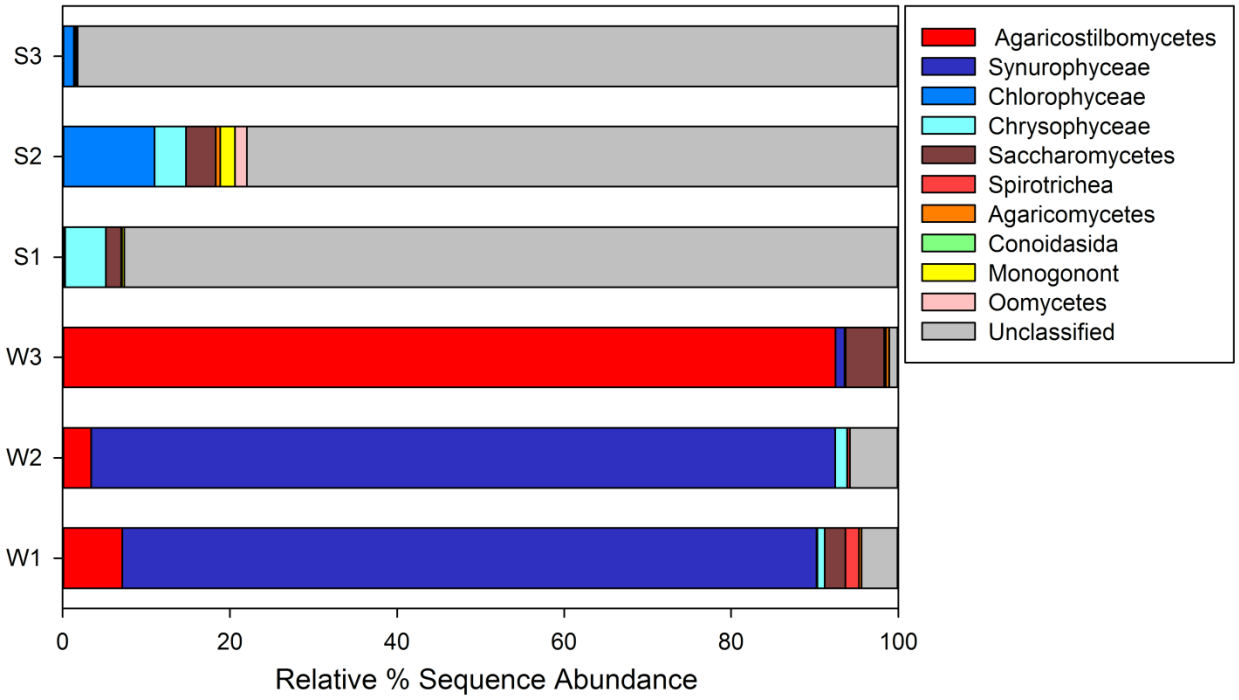

Figure 13: Seasonal Eukaryote AMD community composition depicting class level relative percent sequence abundance.

2.3.4 AMD Community Richness and Diversity

Across all seasonal samples, we found the greatest biodiversity in the Bacterial kingdom, followed by Eukaryotes, then Archaea (Table 3). This pattern was the same for both Chao1 and Shannon estimates of species richness. Chao1 estimates the number of

species present, while the Shannon index estimates both the species diversity and evenness of the community population. Chao1 and Shannon indices for our data also indicate that the average winter Bacterial richness was higher than the average summer Bacterial richness. Species richness was also higher in winter than summer for the Archaeal community, and species richness increase as winter progressed. The Eukaryote richness was found to vary within seasons, but overall Eukaryote richness and species diversity was constant between seasons.

Table 3 Chao1 prokaryote and Eukaryote seasonal richness estimates.

	Bacteria		**Archaea**		**Eukaryote**	
	Chao1	**Shannon**	**Chao1**	**Shannon**	**Chao1**	**Shannon**
W1	835	5.47	30	2.93	33	1.12
W2	1215	5.58	58	3.13	24	0.72
W3	1293	5.60	88	3.57	76	0.63
Winter Average	1114	5.55	59	3.21	44	0.82
S1	876	4.21	--	--	36	0.70
S2	544	3.89	--	--	59	1.71
S3	802	3.96	56	4.47	44	0.97
Summer Average	741	4.02	--	--	46	1.13

2.3.5 Correlations with AMD Water Chemistry

Correlations between water chemistry, microbial community structure/species abundance were examined using multivartiate and redundancy analysis. Four physical AMD properties including pH, conductivity, temperature and ice formation, as well five geochemical AMD properties including iron, copper, sulphate, sulphur, and nickel

concentrations were examined with Bacterial and Eukaryote genera abundances. Overall, Spearman's rank correlation coefficients and P values identified Bacterial genera and Eukaryote class correlations with several AMD properties and metal concentrations shown in Supplemental Table 2. Redundancy analysis (RDA) triplots were also created to show the linear relationship and effects of AMD properties on Bacterial, Archaeal and Eukaryote abundances as well as community structure.

The abundance of *Acidithiobacillus* was found to be negatively correlated with conductivity (Spearman's rank correlation test, $P < 0.05$) and sulphate concentration ($P < 0.01$; Supplemental Table 2). Although statistical significance was week, *Acidiphilium* abundance correlated negatively with pH ($P = 0.07$) and positively with drainage temperature ($P = 0.07$). *Herminiimonas* and *Flavobacterium* abundances both correlated with pH and ice thickness ($P > 0.01$) while negatively correlated with water temperature ($P > 0.05$). *Flavobacterium* abundance was also found to correlate with copper concentrations ($P > 0.05$). *Legionella* and *Leptospirillum* abundances were not found to correlate to any physical or geochemical AMD parameters examined here. *Leptospirillum* abundance only significantly correlated with ice thickness (P value=0.032), and examining the RDA triplot (Figure 14) *Leptospirillum* show similarities to *Acidithiobacillus* (which was expected based on their similar AMD roles), both of which are negatively related to sulphate concentration. The Bacterial microbial community varied throughout the year and attempts to correlate the overall bacterial microbial community profile with different AMD parameters also proved to vary. The winter Bacterial microbial community initially seems to correlate with iron and copper

concentrations, then later W2 with ice thickness, and W3 did not clearly correlate with any parameters measured here (Figure 14). The summer Bacterial community similarly also varied and S1 seems to correlate with drainage temperature, and later (S2) correlates with conductivity, while S3 also was not found to clearly correlate with any parameters measured and is possibly controlled by a set of AMD characteristics

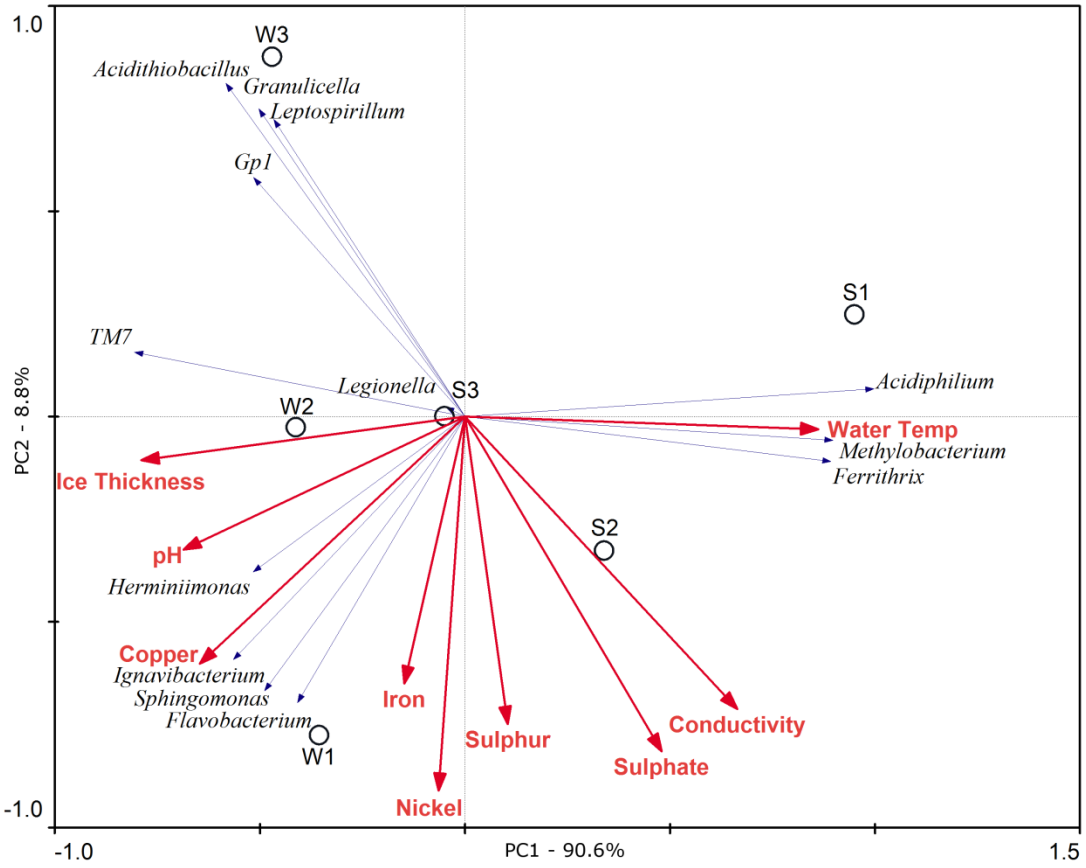

Figure 14: Bacterial redundancy analysis (RDA) triplot, depicting the relationship between the Bacterial AMD community, genus, and AMD chemical and physical properties.

Archaeal and Eukaryote sequencing data could not reliably be classified to genus and thus multivariate analysis was performed on sequencing classified to class, which could

include many genera and species. Multivariate analysis using class abundance and water chemistry was performed on *Thermogymnomonas* and *Ferroplasma* Archaeal classes and both were only found to positively correlate with ice thickness (P value <0.05; due to the simplicity RDA analysis, Archaeal triplots are not shown). Spearnan's rank correlation, depicting Eukaryote genera to physical and chemical AMD parameters are shown in Supplemental Table 2. Except for *Agaricostilbomycetes, Saccharomycetes, and Agaricomycetes*, Eukaryote classes were found to significantly correlate with drainage temperature. *Chlorophyceae*, *Monogonont* and *Chrysophyceae* all positively correlated with drainage temperature and negatively correlated with ice thickness, while *Spirotrichea*, *Synurophyceae* and *Agaricostilbomycetes* were all positively related to ice thickness. Two large fungal classes, *Saccharomycetes* and *Agaricomycetes* were found to negatively correlate with copper, iron, nickel, sulphur and sulphate concentrations as well as conductivity. The Eukaryote RDA triplot (Figure 15) depicts a correlation between the Eukaryote summer AMD community and drainage temperature, where as the winter Eukaryote community is correlated with drainage pH, ice formation, and copper concentrations. *Agaricostilbomycetes* and *Synurophyceae* were found as highly abundant Eukaryote classes, positively correlating with ice formation and *Agaricostilbomycetes* with pH.

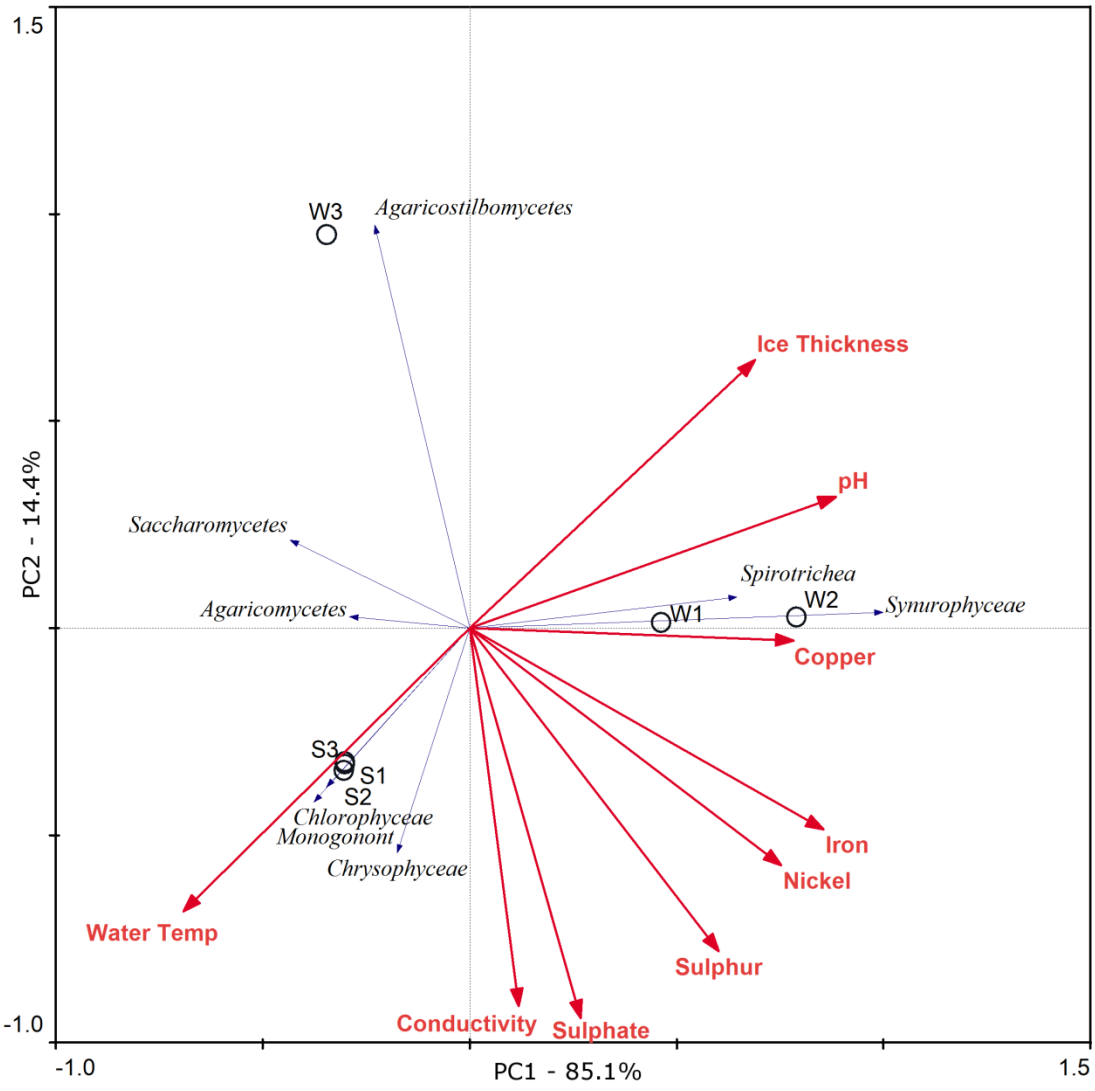

Figure 15: Eukaryote redundancy analysis (RDA) triplot, depicting the relationship between the Eukaryote AMD community, class, and AMD chemical and physical properties.

2.4 Discussion

2.4.1 Copper Cliff AMD Community Profile

We have characterized the Copper Cliff AMD Bacterial, Eukaryote and Archaeal communities across the winter and summer season using direct pyrosequencing. This technique is culture-independent and is expected to give us a more complete view of the seasonal microbial community variation than previous culture-based techniques. The complete AMD microbial community differed significantly between seasons, with Bacteria being the most abundant and diverse group across all samples. Bacterial community variation is significantly different both between seasons, and within seasons, although greater community variation was found between seasons. There was more community variation within the winter than summer and estimates of average species diversity were higher for the winter (1114 OTU's) than summer (741 OTU's). The winter community was largely composed of chemolithotrophs, while in comparison the summer had a high relative abundance of both hetero and chemolithotrophs. The richer winter Bacterial diversity was somewhat unexpected due to the harsh winter conditions (low temp, heavy ice formation, lack of nutrient influx). The richer winter bacterial community may be due to the decreased dominance of *Acidithiobacillus* spp. and increase in opportunistic species. The Eukaryote community was also abundant and diverse thought-out the year, with approximately 60% the DNA collected as compared to Bacteria. Like Bacteria, the Eukaryotes differed significantly between, and within, seasons with larger variation between seasons (Figure 13). Estimated Eukaryote species diversity was similar in both winter and summer (Table 3), although we had expected

greater diversity in the summer in response to increased temperature and organic material. Sequenced Archaeal DNA was generally in lowest abundance of the three domains, although were more abundant than Eukaryotes in the last winter sample (W3 - Figure 3). The Archaeal community profile was relatively similar throughout the year and significant variation between seasons seems to be driven by overall increase in community abundance, specifically at W3. The classified Archaeal community was composed of *Thermogymnomonas* and *Ferroplasma* species, the latter being more commonly isolated in harsh AMD sites and implicated in AMD production (Huang et al. 2011; Edwards et al. 2000).

2.4.2 Bacterial AMD Community

Acidithiobacillus ferrooxidans is a dominant AMD community member at the Copper Cliff site during both summer and winter seasons (57-8% abundance), and the most abundant detected iron oxidizer. *At. ferrooxidans* and *Leptospirillum ferrooxidans* are both considered major contributors to the production of AMD around the world (Kuang et al 2013; Schrenk et al. 1998), although their relative abundance and roles appear to be both site and environment specific (Schrenk et al. 1998). At this site, *Leptospirillum* was only found in rare (<1%) abundance throughout the year, expect for the final winter sample (3.5% abundance) suggesting they play a minor role at this site, consistent with previous work at this site (Auld et al. 2013; Leduc et al. 2002; Rawlings et al. 1999). In contrast, *Acidithiobacillus* was found to dominate the winter months, with an increasing

abundance later in the season, while found in lower relative abundance during S1 and S2 but increased relative abuandacne during S3.

The dominance of *Acidithiobacillus* during the winter months, and reduced abundance of this group in the summer was surprising given that previous work Baker and Banfield (2003) had identified the optimum temperature for *At. ferrooxidans* at 26 ^{o}C. However, other researchers have found large strain-specific differences in optimal growth temperature (Kupka et al. 2007; Jensen and Webb 1995) for this species and it has been found to proliferate at much reduced temperatures as low as 2^{o}C (Mykytczuk et al. 2010; Leduc et al. 1993). Additionally, psychrotolerant strains of *At. ferrooxidans* have recently having been reclassified as *At. ferrivorans* which would tend to dominate AMD environments at cooler temperatures and pH values above 2.3 (Hallberg et al. 2010; Liljeqvist et al. 2011) similar to our winter site. Variation observed in *Acidithiobacillus* species between seasons is possibly due to temperature and pH changes allowing for either *At. ferrooxidans* or *At. ferrivorans* to dominate. The potential dominance of *At. ferrivorans* during the winter correlates with an increase of *At. ferrivorans* seen at W3 when the pH is at the winter low (2.94) and water temp is at the winter high (2.6^{o}C); a more optimal growth range for *At. ferrivorans* than during W1 – W2 (Hallberg et al. 2010; Liljeqvist et al. 2011). This potential change in seasonal abundance of *At. ferrooxidans* and *At. ferrivorans* species could have an effect on the seasonal AMD production (Hallberg et al. 2010), potentially reducing acidification during the winter.

During summer, the acidophilic heterotroph genus, *Acidiphilium* was in highest abundance (87-47%) followed by *Acidithiobacillus* (8 – 36%). The synergistic growth characteristics of *Acidithiobacillus* and *Acidiphilium* have previously been well examined, and the rate of iron oxidation by *At. ferrooxidans* is greater is the presence of *Acidiphilium* spp. (e.g. Lui et al. 2011). *Acidithiobacillus* species are inhibited by organic compounds such as glucose (Marchand and Silverstein 2002) as well as their own iron oxidation by-products such as pyruvic acid (Ishii et al. 2012). While *Acidiphilium* species grow on the organic by products of *Acidithiobacillus*, reducing toxcitiy (Ishii et al. 2012; Harrison 1984) and increasing the growth of both species. The synergistic effect of *Acidithiobacillus* and *Acidiphilium* spp. suggest greater iron oxidation and AMD production during the summer when both genera are in high abundance.

The increased abundance of *Acidiphilium* during the summer correlates with drainage temperature along with an increase abundance of fungal and algae species (Supplemental Figure 5). This increase of *Acidiphilium* may be caused by the influx of organic material produced by Eukaryotes (Das et al. 2009; Nancucheo and Johnson 2012) during the summer. . This might also suggest that lower metabolic activity of the heterotrophic community in the winter would cause a decrease in the abundance of *Acidiphilium* during the winter. In addition to a decrease in organic matter available during winter, *Acidithiobacillus* spp. will have lower iron oxidizing activity at low temperatures (Mykytczuk et al. 2010; Leduc et al. 1993), decreasing organic by products which *Acidiphilium* species metabolize.

Several OTU's classified to rare abundance genera (<1%) including *Ignavibacterium*, *Sulphuricella*, and *Sulphuritalea* were found only during the winter include several species which are facultative anaerobes that oxidize sulphur (Liu et al. 2012; Kojima and Fukui 2011; Kojima and Fukui 2010). The increased biodiversity during the winter may relate to sub-optimal conditions for iron oxidizing species such as *At. ferrooxidans* and *L. ferrooxidans* possibly providing other species, more specifically sulphur oxidizing species to become more dominant. Studies have found similar sulphur oxidizing organism which are cold adapted (Elberling 2005), for example we found winter sequences from *Sulphuricella* spp. which have been isolated from a cold freshwater environment with growth at 0^{o}C (Kojima and Fukui 2010). Additionally, drop in sulphur levels found at the end of winter (W3) could support the theory of sulphur oxidation may become dominant during the winter.

2.4.3 Eukaryote AMD Community

Eukaryote community diversity in AMD has received less attention than either Bacterial or Archaeal AMD communities. Culture independent gene characterization techniques (i.e. direct marker gene sequencing) have indicated that certain AMD sites, such as the Rio Tinto river in Spain, are largely composed of Eukaryotes (Amaral Zettler et al. 2002) while other AMD sites and biofilms contain relatively low Eukaryote abundance (Baker et al. 2009). In the Copper Cliff AMD community, although the highest number of reads were always obtained for Bacteria, the Eukaryote community was highly abundant

throughout the year (Figure 3). The majority of summer sequencing data could not be confidently classified to phylum or class (approx. 90%), highlighing our lack of knowledge specifically on the summer Eukaryote AMD community.

The majority of the winter Eukaryote community was comprised of fungal and algal species belonging to the two phyla Stramenophiles and Basidiomycota. Stramenophiles were comprised mainly of Synurophyceae and related Chrysophyceae (Stoeck et al. 2009) both of which are classes freshwater algae, and Chrysophyceae have previously been identified as a dominant member of an algae AMD community (Amaral Zettler et al. 2002). Synurophyceae algae comprised the dominant portion of the Eukaryote population from W1 to W2 (83 – 89%). Algae have been proposed to increase the growth of both iron and sulphur reducing Bacterial heterotrophs by increasing the amount of dissolved organic carbon (Das et al. 2009; Nancucheo and Johnson 2012). The growth of algae normally occurs at high summer temperatures although algae have been found to proliferate in and about sea ice at greatly reduced temperatures (Schultz 2013). Here the increase of algae occurred during the winter months and did not correlate with organic matter or *Acidiphilium* growth, the major Bacterial AMD heterotroph as expected (Nancucheo and Johnson 2012). Additionally, algae have been proposed to create anoxic zones, promoting the growth of SRB, and although, we did find reduced sulfate concentration at the end of winter, often correlated with SRB (Kolmert and Johnson 2001; Garcia et al. 2001), no significant SRB community was found in the water column. This reduction in sulfate concentrations may be due to an increased abundance of algae and fungal sulfate reducing species similar to previously found (Patron et al. 2008;

Stanislav et al. 2002). Several of these organisms implicated in sulfate reducing belong to *Saccharomycete* Fungal and *Chlorophyceae* algal classes, both of which classes were found in summer and winter AMD samples at our site.

By our last winter sample, W3, the Eukaryote community has shifted to be largely dominated by the fungal phylum, Basidiomycota (93%). Basidiomycota, was mainly comprised of the class Agaricostilbomycetes, a large morphologically and ecologically diverse group of fungi (Bauer et al. 2009) and while fungi are commonly found in AMD systems, their effect on the AMD environment is still not well understood (Das et al. 2009). Fungi are reported to reduce environmental metal concentrations and toxicity allowing for increased growth of Bacterial species; an example is the desulphurization processes by fungi degrading aromatic structures toxic to *At. ferrooxidans* (Bredberg et al. 2002). The large Agaricostilbomycetes class of fungi correlate with the growth of *Acidithiobacillus* (RDA triplot – Supplemental Figure 5) and may allow for increased chemolithotrophic growth (Das et al. 2009). The increased abundance of some fungal AMD species during the winter may decrease metal toxicity that potentially affects some Bacterial species, allowing for the increased species diversity that we found during the winter months. We found the winter Eukaryote community to be largely comprised of algae and fungi. Overall a complex and diverse Eukaryotic microbial community was identified with potential prokaryotic supportive roles at the Copper Cliff AMD site.

Several protists were found in the AMD site, including potential hosts of endosymbiotic Bacteria. We identified members of the phyla Cercozoa, a group that includes many amoeboids (Bass and Cavalier-Smith 2004). Previous AMD studies, including our own, have identified *Legionella*, which have been found in other systems to live endosymbiotically inside the vacuoles of several species of amoedoid protists (Molment et al. 2005). In this study *Legionella* were found as a dominant genus during W3 (8%) and have been identified at AMD sites previously (Auld et al. 2013; Hao et al. 2010). *Legionella* themselves have not been shown to be acidophilic, but the possible endosymbiotic lifestyle and potential association with amooeboid protists, may explain the presence of this tradition neutrophilic group of bacterium in our AMD samples. Decreased temperatures during the winter can cause amoebae to differentiate into cyst (Ohno et al. 2008) from which *Legionella* have been found to survive (Kilvington and Price 1990), possibly explaining the survival of *Legionella* in AMD throughout the year.

2.4.4 Archaeal AMD Community

Archaea have previously been found to be abundant in several other AMD environments (Johnson and Hallberg 2003; Edwards 2000). *Ferroplasma* spp. have specifically received recent attention (Huang et al. 2011; Edwards et al. 2000) as these organisms have been found to oxidize both organic matter and ferrous iron (Dopson et al. 2004) suggesting that they may play a role in the generation of acidic drainage, a process traditionally thought to be controlled by strict chemolithotrophs including *Acidithiobacillus* and *Leptospirillum*. The classified Archaeal sequencing data was

composed of *Ferroplasma* and *Thermogymnomonas* genera. Known members of the genera Thermogymnomonas are acidophilic heterotrophs that grow poorly on iron or sulphur enriched media (Itoh et al. 2007) suggesting they play a limited role in AMD production. We found the highest archael abundance and diversity in our last winter sampling time. At this point, the community included genera of acidophilic heterotrophs, iron oxidizing species, and a high percentage of unclassified Archaea. Interestingly, while *Ferroplasma* spp have been found to dominate in other AMD systems they were rare in this system suggesting substantial variation across systems. In sum, Archaea were a substantial component of the microbial community at some sampling points, but with large seasonal variation and their contribution to AMD, and their overall diversity, is still unknown.

High seasonal microbial community variation found at our AMD site suggests potential variability in the acidification process throughout the year. Variation in community profiles and presence or absence of members may indicate differing degrees of AMD production seasonally. Differences in Bacterial, Eukaryote, and Archaeal seasonal communities seem to be driven by a large number of geochemical AMD properties that vary greatly throughout the year. Bacteria, specifically *Acidithiobacillus* spp. have largely been largely implicated in the production of AMD, and were found here to vary greatly in relative community composition throughout the year. Recent work also suggests that Archaea and Eukaryote species may to be implicated in iron oxidation, for example species of the Archaeal genus *Ferroplasma* were found here in varying degrees

throughout the year and potentially suggest varying seasonal AMD production from different sources and mechanism.

We found a high rate of community change between sampling dates for Bacterial and Eukaryote communities and further identify high AMD seasonal community variability previously found (Leduc et al. 2002; Edwards et al. 1999). High community variability also highlights problems with single sample characterization studies which try to examine community structure and dynamics based only on one community profile which may or may not be similar throughout the year, highlighting the need for additional characterization time points.

Chapter 3

3 Future Directions

3.1.1 Overview

One of the largest ecological problems facing the mining industry today is the production of acid mine drainage (Hao 2010). Considerable AMD research has, and continues to be, focused on characterizing the microbial community that inhabits this environment and understanding the role these microbes play in the production of AMD, with an ultimate goal of prevention and remediation. Traditionally, the AMD microbial community was thought to be simple, controlled exclusively by iron oxidizing chemolithotrophs. Recently, research has identified a suite of different microbes (Dopson et al.2004, Auld et

al. 2013) that potentially could all play roles in the AMD environment, including additional ferrous iron oxidizing microbes such as the Archaeal *Ferroplasma* species (Dopson et al. 2004) that have been identified as the dominant prokaryote in some AMD environments (e.g. Edwards et al. 2000), and were present at varying seasonal time points in other studies (Huang et al. 2011; Edwards et al. 1999). *Ferroplasma* was found at our AMD; although not in high abundance (Chapter 2). The presence of this group of Archaea at our site, and the potential of this group to play a role in AMD production, highlights the need for a better, more complete, community characterization.

I characterized the AMD microbial community using direct sequencing and found significant variation in the seasonal microbial community. The prokaryote and Eukaryote microbial community changed significantly within just two weeks, the shortest time period in this study. The speed at which the microbial community changes, and the amount of variation that occurs between different seasons or time points, is still not well understood, however my research suggests a large amount of temporal variation and quick turnover of species. Further work is needed to determine the rate of microbial AMD community turnover; direct pyrosequencing of the small ribosomal subunit could still be used utilized to identify the community at additional time points within a season (daily, weekly). The presence of significantly different AMD community profiles between seasons suggests that the community structure changes as microbes and communities more suited to specific environmental conditions become dominant. Greater understanding of community dynamics and variation could identify not only additional species potentially playing roles in AMD systems, but would almost

undoubtedly lead to greater understanding of the acidification process throughout the year. Here I sampled over summer and winter seasonal periods, but did not examine across spring and fall. Obtaining community profiles throughout the year, perhaps monthly, would better determine seasonal trends. Additional samplings could potentially address not only the number of different microbial community profiles that exist throughout the year, but the speed at which the communities change, and whether the community profiles correlate with the four seasons or at varying times throughout the year.

3.1.2 Further Characterizing Seasonal Variation

Variation in the microbial community across different seasons is not a new topic, but seasonal microbial community variation specifically at AMD sites is still understudied and poorly understood (Huang et al. 2011; Tan et al. 2009; Edwards et al. 1999). In my thesis research, I assessed the seasonal variation in AMD microbial communities by comparing samples from 6 distinct time points: three samples collected during the summer (collected every 2 weeks) and three samples collected during the winter (also every 2 weeks). I found significant community variation both between seasons and between two week periods within a season. Interestingly, I found several seasonal trends, e.g. a chemolithotrophic winter Bacterial profile, but heterotrophic and chemolithotrophic summer Bacterial profile, and an increasing Archaeal population throughout the winter. Additionally, the majority of the eukaryotic sequences from the summer collection dates could not be classified, whereas a majority of the sequences from the winter were

confidently assigned at the species level, largely fungal and algal species. Similarly, approximately 25 - 50 % of the Archaeal sequences could not be classified to either phyla or class, highlighting how little we know about the Archaeal community. This large percentage of unidentified taxa suggests that there may be seasonal communities that are underrepresented in genomic databases that are not fully understood. Similar AMD characterization studies have recently found species that were previously thought to play only a minor role in AMD but are, in fact, in high abundance in some locations (*e.g.* *Ferroplasma* has been implicated in AMD production – Bond et al. 2000; Dopson et al. 2004). Together these results highlight the diversity of microbial communities and the importance of continued research into characterizing the AMD community at specific seasonal time points from which less knowledge on the community is known, (*e.g.* summer Eukaryotes and winter Bacterial). Future studies could focus on identifying unknown community members and temporal variation or trends. Sampling from AMD sites on a weekly basis, rather than a bi-weekly basis, as well as sampling monthly through all seasons could provide more information on the rate of turnover and community difference throughout the year.

3.1.3 Site Specific Microbial Variation

The presence of organisms is not uniform within a habitat or community and different sub-habitats occur in all environments including AMD sites, e.g the acidified drainage, biofilms, or sediment samples all of which contain different communities (Schrenk et al. 1998). My research shows that seasonal geochemical and site specific changes are

correlated with microbial AMD community change. Here I examined the prokaryote and Eukaryote community variation within pooled drainage samples, and found significant community change across 2 week periods. Variation in community composition between locations (spatial community variation) has received little attention in AMD research. Variation has been found, however, in the studies that have been done. A series of studies examining the Bacterial communities from sites, meters to hundreds of kilometers apart, all suggest different communities (Huang et al. 2011; Tan et al. 2009). Previous AMD spatial work has been done on community variation using highly variable geochemical locations, all of which are expected to have significantly different communities (Huang et al. 2011; Tan et al. 2009). Future work could address spatial community variation within a more homogeneous site. This work could test for correlations in community structure with geochemisty. A seasonal component could also be added addressing the potential for greater or lesser variation across locations and seasons, e.g. asking are some sites more seasonally variable? Many examples exist of soil communities, in which significantly different communities are found meters apart in largely homogeneous sites (Saetre and Baath 2000). Instead of taking several drainage samples from different pond locations and pooling samples, as was done in this project, separate pond locations could all be characterized in combination with seasonal characterization. Similarly we would expect to find different AMD drainage communities based on location within a pond, possibly related to water outflows, heavy mineral deposits, and pond depth etc. but the actual extent of this variation is unknown. The drainage pond at the Copper Cliff site contains regions of varying depth, stagnant

and flowing locations, and sites with higher possible mineral precipitation based on sediment color, all of which could be used for site specific sampling and characterization.

3.1.4 AMD Community and Bioremediation

Many different treatment options exist for the alkalinization and disposal of metals from acid mine drainage, as briefly reviewed in Chapter 1. Processing of AMD at the Copper Cliff location is performed using a mechanically agitated reactor (MEND 2005) where the drainage is mechanically mixed with lime to increase the pH. Many treatment systems still do not utilize mechanical agitated reactors to mix the AMD and lime, but rather allow the wastes to enter a stream and eventually a settling pond where lime treatment is used. The use of both reactors and natural pond formations for the neutralization and precipitation of metals from acidic drainage could be improved by a better understanding of the microbes and their role in AMD production. Iron and sulphur oxidizing Bacteria are the cause of drainage acidification. Iron and sulphur reducing Bacteria that reduce the acidity, are also present in AMD systems but in lower abundance. The use of these naturally occurring microbes that reduce iron and sulphur, and increase pH, in the cleanup of acidic drainage is appealing due to the potential for sustained microbial action, non-invasiveness, and low cost (Glick 2010). While not directly related to bioremediation of AMD sites, a better understanding of the acidifying microbial community may allow us to more effectively harness the reducing microbes in the cleanup of AMD. The oxidizing microbial community (acidifying community), their interactions with other community members, seasonal variation in the production of

AMD, and community dynamics could all affect the use of reducing microbes in remediation purposes (e.g. species specific interactions with may differ depending upon season). My research has identified a more complex and dynamic AMD community than previously documented, increasing our knowledge of the microbes potentially important in the acidification process occurring at AMD sites.. In addition to seasonal abundance and species specific variation, I found a diverse Eukaryote population and Eukaryotes have been suggested to play supportive role for prokaryotes (Baker et al. 2009; Baker et al. 2004). Eukaryotes, along with the Archaeal and Bacterial species, potentially play an important role in AMD environment that have previously not been well examined. The complexity of microbes found, high seasonal species variation and high fluctuation in relative species abundance suggest that AMD production may vary seasonally and that many different mechanisms of AMD production may be possible. Future work could focus on the modification of pre-existing AMD communities, or even the production of artificial microbial communities aimed at the bioremediation of environmental AMD sites. Neither method is likely possible without a better understanding of AMD production mechanisms and the microbes that play direct, or supportive roles in AMD production. A possible next step is a complete understanding of the species-specific roles of microbes, and pinpointing the species responsible for the removal of toxic metals, chemicals, or even the alkalinization of sites such as SRB. Artificial microbial communities have previously been constructed (e.g. Baffan-Dubau et al. 2001) and this approach could be adapted for bioremediation with continued AMD community analyses. The production of artificial microbial communities may be difficult *in situ* due to incompatibility with the pre-existing community, but could be possible in man-made

sterile tanks, such as those currently used in the neutralization of AMD. Advances in molecular community characterization techniques are allowing for more complete community profiles (Mohapatra et al. 2011) and better sequencing technology and metagenomics analyses will hopefully soon identify new AMD gene functions and species specific AMD roles. Given the current pace of advancements in our understanding of AMD microbes, we may soon be able to produce artificial microbial communities or modify microbial communities aimed at AMD bioremediation.

3.1.5 AMD Sediment Community

The microbial AMD sediment community, and the effect of sediment microbes on the production of AMD or its influence on the drainage community, has received some, but limited attention (Sanchez-Andrea et al. 2012). The seasonal community characterization performed here has identified drainage species that vary in abundance between seasons, ranging from non-existent at one time point to dominant organisms at another. This variation throughout the year, including variation from absent to dominant, could be potentially related to the upper sediment layers harboring dormant organisms between periods of optimal conditions.

Future work to characterize the AMD sediment community could examine the sediment community's potential function as a biological sink, harboring species between optimal drainage conditions and may explain the drainage community changes throughout the

year. Additionally, the characterization of the AMD sediment by direct sequencing could provide information and even uncover novel sulfate reducing Bacteria (SRB) and iron reducing Bacteria (FeRB). Sulfate and iron reducing anaerobic Bacteria found in the sediment layers of AMD sites are potential bioremediation organisms (Johnson and Hallberg 2005). Additionally, AMD sediment work has found representatives from the gammaproteobacteria, many of which are capable of sulfate reduction (Hao et al. 2007), as well as the expected iron and sulphur oxidizing species (Sanchez-Andrea et al. 2012; Hao et al. 2007). Although the methods previously used were culture independent, they did rely on species-specific probes, cloning techniques, and previous information on AMD microbes (Sanchez-Andrea et al. 2012; Sanchez-Andrea et al. 2011; Lucheta et al. 2013); to my knowledge no previous direct sediment sequencing examination has been performed. Variation in the microbial community across sediment layers, e.g. as we move deeper, is of particular interest because of potential SRB and FeRB in the deeper anoxic layers. Previous culture independent work has identified both members of FeRB (*Acidithobacillus* spp.) and SRB (*Desulfosporosinus* and *Desulfurella* spp) as dominant members of the sediment community (Sanchez-Andrea et al. 2012). Other locations examined also utilizing culture independent techniques have found the AMD sediment community to be largely composed of Chloroflexi (green non-sulphur reducing bacteria) and uncultured heterotrophs (Lucheta et al. 2013), highlighting the lack of consensus and understanding of AMD sediment communities or their great variability. Potential community variation of sediment communities may potentially correlation with physical sediment characteristics, such as metals, and oxic/anoxic zones. Variation is high between layers of soil (Frouz et al. 2001), suggesting that variation will also be high

across the depth of sediment. Future work could also address the amount of sediment community variation within a site, similar to the above AMD proposed site variation work. Sanchez-Andrea et al. (2012) found the benthic AMD community to differ between sediment sites, while the sediment community is expected to change based on site specific variation (Herr and Gray 1997). The overall community structure, I theorize, should stay more spatially and seasonally constant compared to surface layers or drainage (similar results found in soils – Blume et al. 2002) due to the less dynamic conditions within the sediment compared to the drainage waters. Characterizing the sediment community, including the different SRBs and FeRBs, will not only increase the number of potential microbes we can utilize in bioremediation, but also hopefully result in a more complete understanding of seasonal variation in AMD production.

3.2 Conclusion

Identifying the complete microbial community, and the roles and interactions across the community members in the production of AMD, is a possible first step in novel approaches to bioremediation and control of such sites. Here I characterized the seasonal AMD microbial community variation at Vale's tailings facility (Copper Cliff, ON, Canada) using culture independent direct sequencing. Significant community variation was found both between and within seasons of all three microbial domains. Bacteria dominated the AMD microbial community throughout the year, with higher taxonomic diversity during the winter than summer. Members of the genus, *Acidithiobacillus* dominated the winter and members of the genus, *Acidiphilium* in the summer.

Interestingly, both Eukaryotes and Archaeans also make up substantial fractions of the AMD microbial community and likely also contribute to the community metabolism that drives AMD production, either directly or indirectly. Eukaryotes were composed of fungal and algal species during the summer while the eukaryotic microbes found in the winter were largely unclassified. Archaeans were in relatively low abundance and may play only a minor role at this site, however, were more abundant than Eukaryotes by the end of the ice over season. The microbial community composition is likely driven by physical and geochemical site properties that vary throughout the year, specifically temporal variation. Temporal variation between seasons correlates with both prokaryotic and eukaryotic growth, specifically acidophilic heterotrophs which directly affect iron oxidizing species. The weekly community turnover and diversity found at the Copper Cliff AMD site suggests a complex and extremely dynamic microbial community controlled by seasonal and geochemical properties that change weekly.

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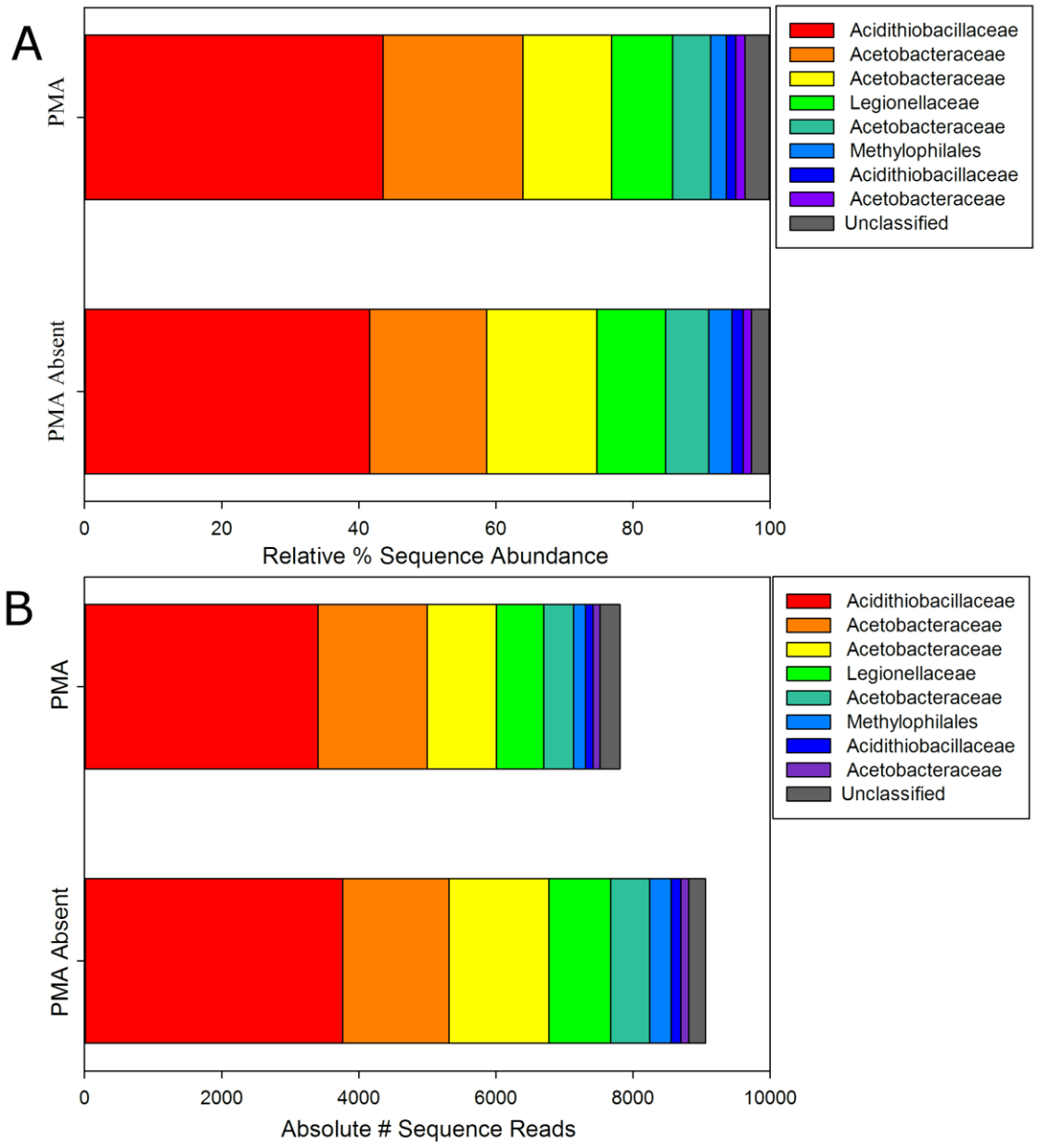
Appendix A

Supplemental Table 1 Water chemistry analysis for six sampling times; three summer and three winter dates.

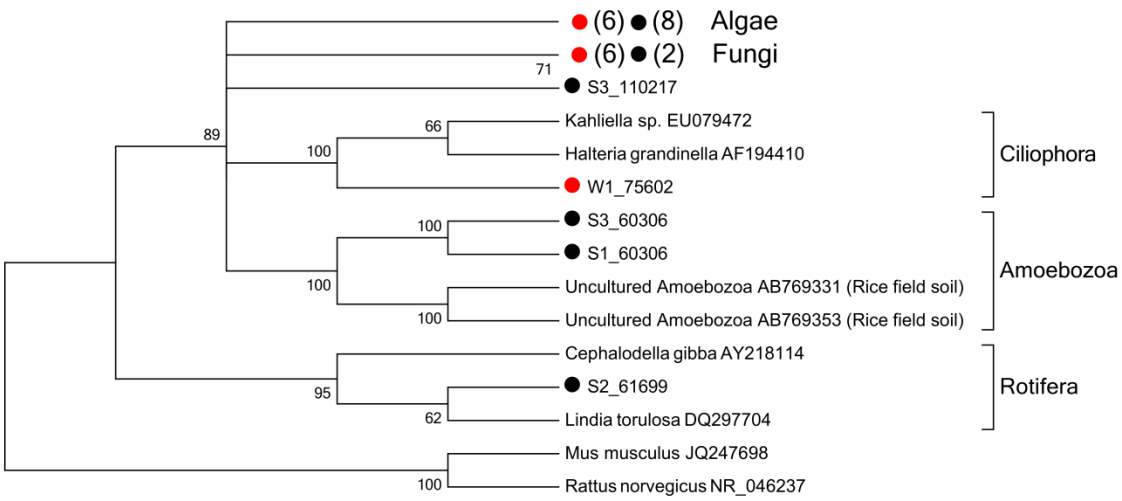
Parameter	W1	W2	W3	S1	S2	S3
pH	3.27	3.28	2.94	2.76	2.75	2.80
Water Temp (^{0}C)	1.1	1	2.6	22.8	24.7	18.1
Ice Thickness	20	15	15 inch	---	---	---
M-Alkalinity (pH 4.5) as CaCO3 (mg/L)	<1	<1	<1	<1	<1	<1
Conductivity (µS/cm)	4020	3670	2070	4430	4510	3560
Bicarbonate (mg/L)	<1	<1	<1	<1	<1	<1
Calcium (ug/L)	423000	398000	87700	490000	317000	272000
Carbonate (mg/L)	<1	<1	<1	<1	<1	<1
Sulphate (mg/L)	3080	2770	1240	3030	3060	2870
Sulphur (ug/L)	817000	942000	307000	710000	749000	820000
Phosphate (mg/L)	<1	<10	<10	<1	<10	<10
Total Phosphorus (as P)	0.0236	0.015	0.017	0.0222	0.013	0.011
Total Aluminum (ug/L)	29900	29900	12000	20800	15000	23300
Total Antimony (ug/L)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Total Arsenic (ug/L)	3.4	3.4	1.9	2.4	2	2
Total Barium (ug/L)	14	12.5	7.6	6.9	7	6
Total Beryllium (ug/L)	1.6	2	<0.5	0.73	1.2	0.7
Total Bismuth (ug/L)	<1	<1	1.1	<1	<1	<1
Total Boron	180	222	23	322	233	214

(ug/L)						
Total Cadmium (ug/L)	9.77	12.2	1.57	5.78	6.9	5.8
Total Calcium (ug/L)	433000	468000	95100	518000	317000	296000
Total Cerium (ug/L)	906	605	147	533	429	645
Total Cesium (ug/L)	<1	<1	<1	<1	1	<1
Total Chromium (ug/L)	23.2	20.8	64.1	16.3	14	30.5
Total Cobalt (ug/L)	1180	1060	202	684	634	640
Total Copper (ug/L)	2350	1960	1150	944	1020	1750
Total Europium (ug/L)	15	11.7	2.1	8.6	12	10
Total Gallium (ug/L)	<1	<1	<1	<1	<1	<1
Total Iron (ug/L)	431000	558000	<20	266000	199000	304000
Total Lanthanum (ug/L)	407	305	67.3	263	361	315
Total Lead (ug/L)	2.1	1.2	6	<1	2	<1
Total Lithium (ug/L)	128	131	24	115	118	95
Total Magnesium (ug/L)	141000	127000	28700	195000	166000	147000
Total Manganese (ug/L)	4560	4570	1090	5460	4270	4480
Total Mercury (ug/L)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Total Molybdenum (ug/L)	<1	<1	5.1	<1	<1	<1
Total Nickel (ug/L)	44200	40300	10800	26700	33400	27000
Total Niobium (ug/L)	<1	<1	<1	<1	<1	<1

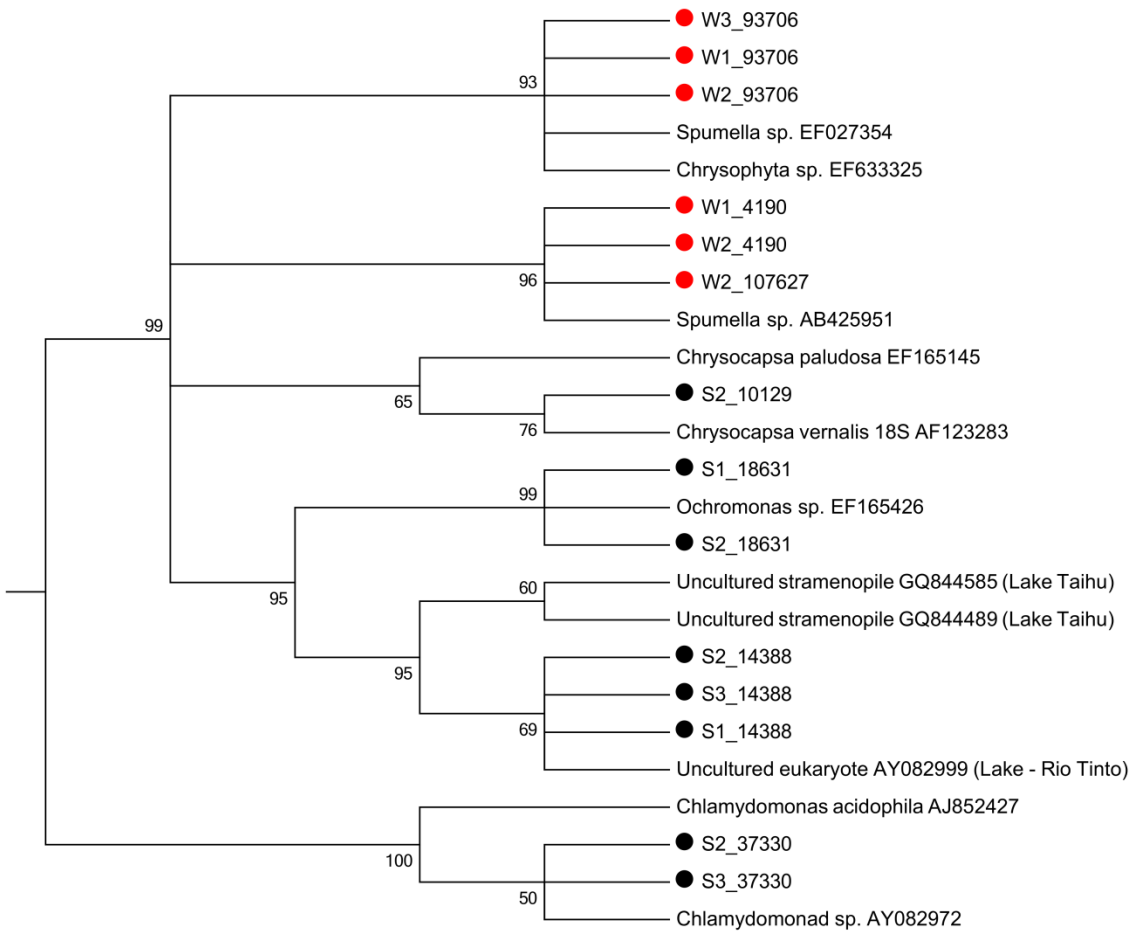
Total Potassium (ug/L)	40900	37300	5000	43700	37400	34400
Total Rubidium (ug/L)	80.7	78.7	9.2	75	73	57
Total Scandium (ug/L)	12.1	14.3	2.8	9.6	6	8
Total Selenium (ug/L)	14.2	11.5	4.5	5.4	5	7
Total Silicon (ug/L)	19300	19000	4200	19000	25000	17000
Total Silver (ug/L)	<0.1	0.2	<0.1	<0.1	<0.1	<0.1
Total Sodium (ug/L)	122000	140000	16900	188000	124000	123000
Total Strontium (ug/L)	1100	665	170	1340	1130	1320
Total Tellurium (ug/L)	<1	<1	<1	<1	<1	<1
Total Thallium (ug/L)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Total Thorium (ug/L)	9.8	9.2	2.5	4.5	5	7
Total Tin (ug/L)	<1	<1	<1	<1	<1	<1
Total Titanium (ug/L)	7.1	9.6	18.6	6	7	6
Total Tungsten (ug/L)	<1	<1	<1	<1	<1	1
Total Uranium (ug/L)	19.2	20.7	3.5	14.5	19	16
Total Vanadium (ug/L)	6.2	5.2	22.2	1.4	<1	1
Total Yttrium (ug/L)	313	297	39.9	188	252	192
Total Zinc (ug/L)	1190	970	119	672	910	640
Total Zirconium (ug/L)	<1	<1	<1	<1	<1	<1



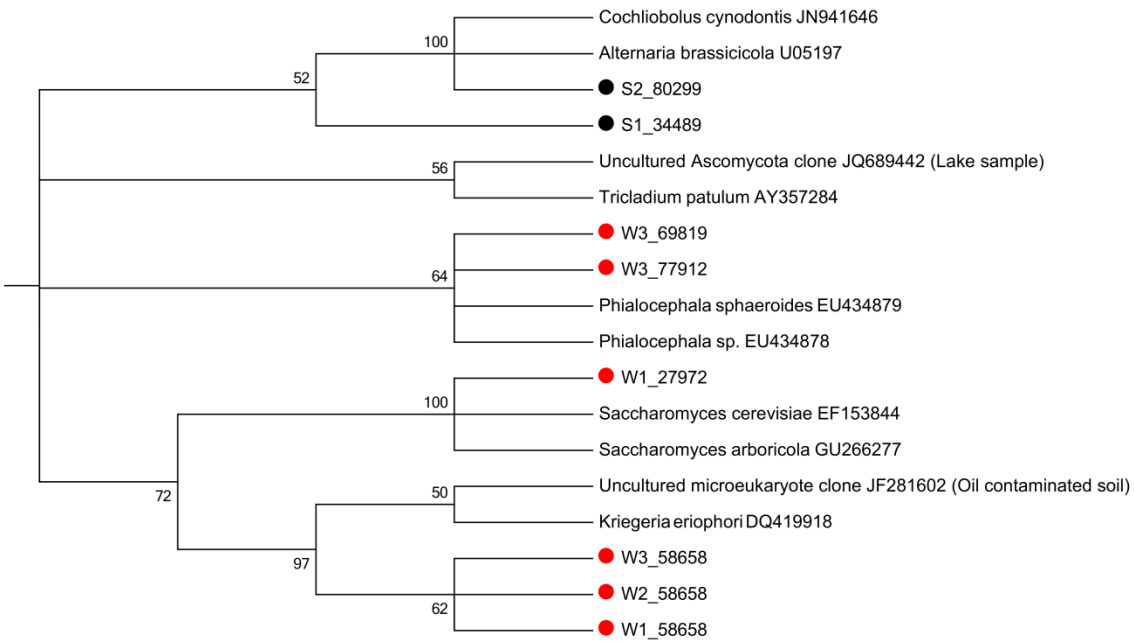
Supplemental Figure 1: PMA and Non-PMA treated Bacterial AMD community profiles from S3 (Order). Figure (A) depicts relative percent sequence abundance and figure (B) depicts absolute number of sequence reads.



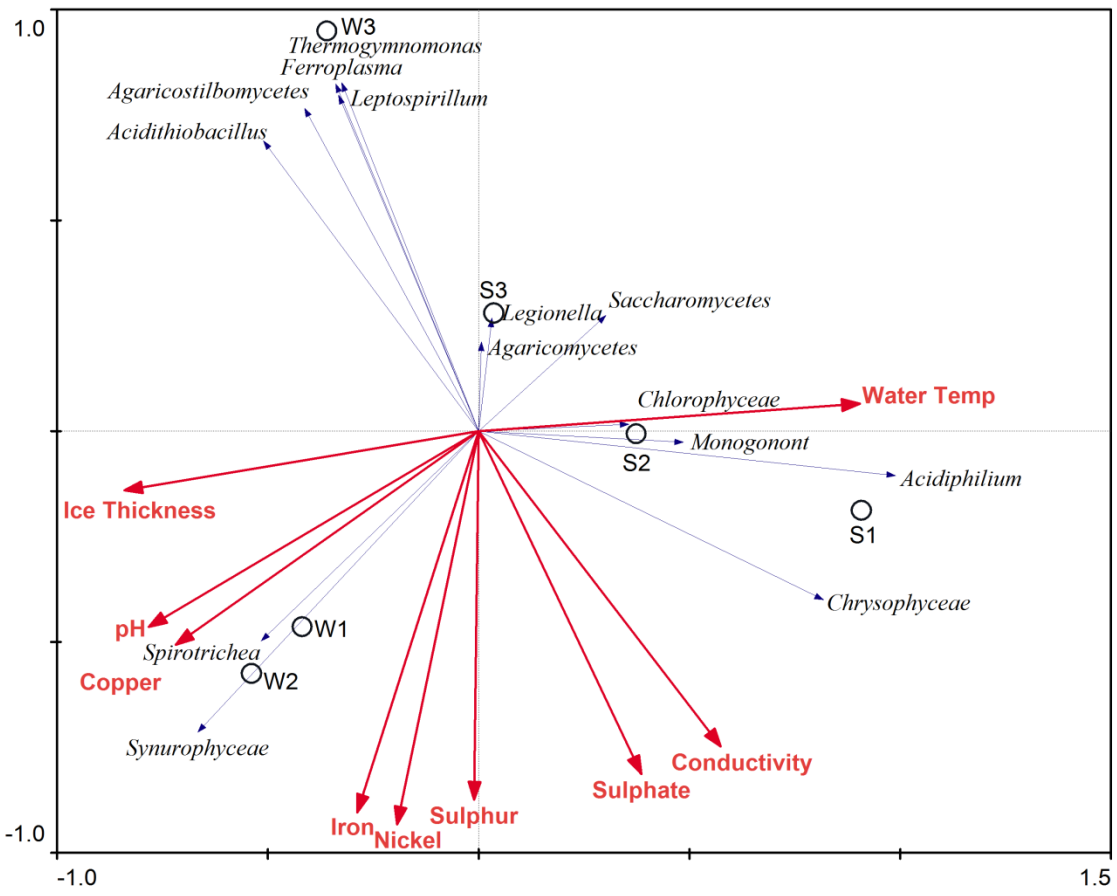
Supplemental Figure 2: Maximum likelihood condensed tree of dominant (>1%) Eukaryote sequencing OTU's with bootstraps >50% including top two closest BLAST hits. Red symbols indicate winter OTU's while black symbols represent summer OTU's. Algae and fungal clades have been compressed and are shown in supplemental figures 2 and 3.



Supplemental Figure 3: Expanded algae maximum likelihood tree of dominant (>1%) Eukaryote OTU's. Red symbols indicate winter OTU's while black symbols represent summer OTU's.



Supplemental Figure 4: Expanded fungal maximum likelihood tree of dominant (>1%) Eukaryote OTU's. Red symbols indicate winter OTU's while black symbols represent summer OTU's.



Supplemental Figure 5: Redundancy analysis (RDA) triplot, including Bacterial and Eukaryote AMD community (circular symbols), species (black arrows), and AMD chemical and physical properties (red arrows).

Supplemental Table 2 Spearman's rank correlations analysis between species abundance and seasonal physical and geochemical properties of Vale's Copper Cliff AMD site.

		pH	Water Temperature	Conductivity	Ice Thickness	Iron	Sulphur	Sulphate	Nickel	Copper
Bacteria	*Acidithiobacillus*			-0.89 (0.019)				-0.94 (0.0048)		
	Acidiphilium	-0.77 (0.072)	-0.77 (0.072)							
	Leptospirillum				0.83 (0.039)					
	Herminiimonas	0.94 (0.0051)	-0.94 (0.0051)		0.89 (0.019)					
	Flavobacterium	0.88 (0.02)	-0.88 (0.02)		0.98 (0.0004)					0.82 (0.046)
Archaea	*Thermoplasmata*		0.85 (0.034)							
	Ferroplasma		0.89 (0.019)							
Eukarya	*Spirotrichea*	0.93 (0.0077)	-0.93 (0.0077)		0.94 (0.0054)					0.81 (0.0499)
	Conoidasida						-0.88 (0.021)			-0.88 (0.021)
	Monogonont	-0.94 (0.0051)	0.94 (0.0051)		-0.89 (0.019)					
	Synurophyceae	1 (0.0001)	-1 (0.0001)		0.83 (0.039)					0.83 (0.042)
	Oomycetes	-0.94 (0.0051)	0.94 (0.0051)		-0.89 (0.019)					
	Chrysophyceae			0.87 (0.024)						
	Chlorophyceae	-0.89 (0.019)	0.89 (0.019)							
	Agaricostilbomycetes				0.89 (0.019)					

*P-Values (brackets) provided are those that represent significant correlations. Values represent Spearman's rank correlation coefficient, in which values of +1 or -1 indicated a perfect correlation between variables.